Università degli Studi di Palermo

AREA QUALITÀ, PROGRAMMAZIONE E SUPPORTO STRATEGICO
SETTORE STRATEGIA PER LA RICERCA
U. O. DOTTORATI

Dottorato di Ricerca in Scienze Agrarie, Alimentari e Forestali (D071)
Dipartimento di Scienze Agrarie, Alimentari e Forestali
SSD AGR/16 – MICROBIOLOGIA AGRARIA

BIOTECHNOLOGICAL INNOVATIONS IN FERMENTATION PROCESS OF BREWING AND HONEY-BASED BEVERAGES INDUSTRY

IL DOTTORE
MICHELE MATRAXIA

IL COORDINATORE
PROF. VINCENZO BAGARELLO

IL TUTOR
PROF. GIANCARLO MOSCHETTI

CO TUTOR
PROF. NICOLA FRANCESCA
PROF. ALDO TODARO

CICLO XXXIV
ANNO CONSEGUIMENTO TITOLO 2022

Summary

ABSTRACT 2
CHAPTER 1 4
General introduction 4
1.1 Brewing history and technology 5
1.2 Mead history and production 7
1.3 Fermentative microorganisms: yeasts and bacteria 9
1.4. Yeast nutrition 13
1.5. Fermentative cultures inoculum strategy 14
1.6. References 16
CHAPTER 2 21
Non-conventional yeasts from fermented honey by-products: focus on Hanseniaspora uvarum strains for craft beer production 21
2.1. Introduction 22
2.2. Materials and methods 24
2.3. Results 32
2.4. Discussion 48
2.5. Conclusion 51
2.6. References 52
CHAPTER 3 57
Technological screening and application of Saccharomyces cerevisiae strains isolated from fermented honey by-products for the sensory improvement of "Spiritu re fascitrari", a typical Sicilian distilled beverage 57
3.1. Introduction 58
3.2. Material and methods 59
3.3. Results and discussion 65
3.4. Conclusions 76
3.5. References 76
CHAPTER 4 80
Use of Saccharomyces and non-Saccharomyces strains isolated from honey by-products to improve and stabilize the quality of mead produced in Sicily 80
4.1. Introduction 81
4.2. Materials and methods 82
4.3. Results and discussion 86
4.4. Conclusions 94
4.5 References 94
CHAPTER 5 98
General conclusions 98
CHAPTER 6 101
List of publications 101

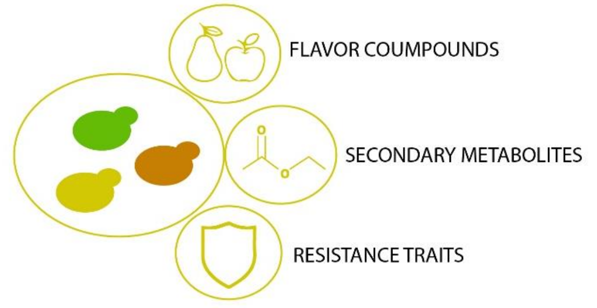
ABSTRACT

La ricerca di microrganismi fermentanti in grado di migliorare le proprietà microbiologiche, fisiche, chimiche, sensoriali e organolettiche delle bevande alcoliche fermentate come vino, birra, idromele, sidro, è considerata ad oggi un punto chiave per lo sviluppo del settore.

Negli ultimi anni, l'aumento della domanda al consumo di nuove bevande fermentate ha causato un enorme bisogno di tecnologie di produzione innovative al fine di ottenere diverse tipologie di prodotti caratterizzati da proprietà sensoriali peculiari. Per raggiungere tale obiettivo, l'impiego di nuovi microrganismi, sia fermentanti che non fermentanti, in grado di conferire al prodotto caratteristiche organolettiche peculiari ed uniche, può rappresentare una valida alternativa ai classici ceppi attualmente in commercio. Il settore vitivinicolo-enologico risulta quello in cui la ricerca ha sviluppato conoscenze sufficienti ad applicare nuove specie e nuovi ceppi di lieviti su scala industriale. La ricerca di lieviti non convenzionali applicabili alle bevande alcoliche che nell'Europa meridionale sono considerate secondarie rispetto al vino, ovvero birra, sidro ed idromele, risulta essere ancora poco investigata, essendo scarsi gli studi disponibili sulla fermentazione di queste bevande. In particolare, il settore birrario sta riscoprendo un rinnovato interesse nei confronti di lieviti isolati da matrici territoriali, considerati responsabili del miglioramento e della creazione di bouquet particolari, che consentono di ottenere produzioni legate al territorio e differenziate su un mercato in forte espansione.

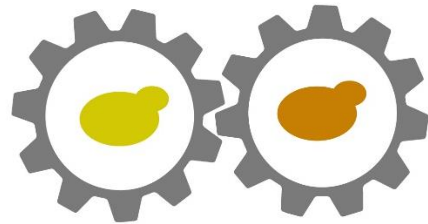
Analogamente alla produzione di birra artigianale, la produzione di idromele sta attualmente vivendo un interesse a livello amatoriale e professionale in tutto il mondo. Sebbene non sia una bevanda molto popolare, il suo consumo in Europa è in costante crescita. Anche in questo settore la letteratura scientifica risulta limitata, specialmente in ambito microbiologico, dato che per condurre le fermentazioni alcoliche sono spesso usati ceppi di *S. cerevisiae* di origine enologica. Diversamente dal mosto d'uva, il mosto-miele risulta carente in fattori nutrizionali che limitano la crescita microbica. Per tale motivo, la ricerca di lieviti appropriati in grado di crescere e fermentare queste matrici risulta essere un punto cruciale per mantenere un elevato standard qualitativo di questa bevanda.

Sulla base di queste premesse, durante il triennio di Dottorato di ricerca è stata condotta uno studio inerente: l'ecologia microbica di matrici zuccherine; l'isolamento, la caratterizzazione genotipica e tecnologica di lieviti, sia *Saccharomyces* che non-*Saccharomyces*; la loro applicazione, sia singolarmente che in combinazione, durante le fermentazioni sperimentali di mosto di birra e mosto-miele; la valutazione della cinetica di fermentazione, metaboliti secondari, composti organici volatili ed analisi sensoriale dei prodotti ottenuti.



RETHINKING NOVEL BEVERAGES

NON - CONVENTIONAL YEASTS
DISTINCTIVE METABOLIC PROFILE



SPECIALTY & DISTINCTIVE PRODUCTS

INNOVATIVE PROCESSES

graphic Stefano Lo Voi

CHAPTER 1

General introduction

1.1 BREWING HISTORY AND TECHNOLOGY

Beer is one of the oldest and most widely consumed alcoholic drink in the world. It is brewed from cereal grains, most commonly from malted barley, though wheat, maize, and rice are also used. Through alcoholic fermentation, with *Saccharomyces cerevisiae* or *Saccharomyces pastorianus* (syn. *Saccharomyces carlsbergensis*) yeast strains, the sugars are transformed into ethanol and carbon dioxide (Legras et al., 2007).

Most modern beer is brewed with hops, which add bitterness and other flavours and act as a natural preservative and stabilizing agent. Other flavouring agents such as gruit, herbs, or fruits may be included or used instead of hops.

Beer origins date back to long ago. The first evidence in the history of the preparation of a beer-like drink was by the Sumerians, the inhabitants of the fertile strip of land between the Tigris and the Euphrates, and date back to about 6,000 years ago (Katz and Maytag, 1991).

However, the ways of managing the drink were different. Mesopotamia, for example, was the land that first saw the brewer profession rise. The labours of workers for its preparation were paid in beer. At that time, various types of beer were produced: dark, light, red, strong, sweet and aromatic and it seems that there were twenty types of beer available on the market in Babylon, the richest city in Mesopotamia. The beer also had a religious and ritual significance, in fact it was drunk during the funeral to celebrate the virtue of the deceased and was offered to the divinity to guarantee a peaceful rest to the dead. Beer was of similar importance in Ancient Egypt where the subjects of the pharaohs used to drink this beverage from childhood, also used for food and medicine (Arnold, 1911).

Until the Middle Ages, the brewing process was the prerogative of women only. Slowly this prerogative vanished as beer began to be produced in monasteries; this art was adopted by the monks (first and foremost Belgian and Dutch) to keep alive the link between beer and religion. The first Babylonian women who produced beer were in fact the temple's priestesses.

Gradually, brewing became a purely male activity. Before long, the monks began to produce much more than necessary, and therefore began to sell their surplus. Unfortunately, the rulers of the time sensed the possible profits that could be made on the beer trade, and pushed to prevent the monks, who paid no taxes, from operating in such a profitable field (Meussdoerffer, 2009).

Over the time, the hop was introduced into brewing by replacing the "Grut", a mixture made of juniper berries, blackthorn, oak bark, absinthe, anise, rosemary, etc., herbs that were later considered also dangerous (Horseny, 2003).

In 1516 the Duke of Bavaria established that only barley (including barley malt), hops and pure water could be used to make beer.

With the use of hops, the drink reveals a flavour similar to today's beer, although the use of yeast was still unknown and fermentation was considered a random process.

Only in the 16th century, brewers discovered how to successfully control barley fermentation, making improvements in terms of quality and quantity.

The current beer production process has been achieved thanks to many changes made over time and involves the following steps:

- Malting: malt is obtained mainly from barley but also from other cereals. However, barley has always been the most used cereal to produce beer for several reasons: high quantities of starch, good organoleptic qualities and low percentage of fats (Giardini and Baldoni, 2000).

  The malting process involves:
  - Steeping: barley or other cereals are cleaned and placed in the maceration tanks where they are hydrated and oxygenated to facilitate germination.
  - Germination: in this phase, the embryonic development takes place, after that the bud starts to feed on the starchy substances of the endosperm, which determines their rupture and the formation of the enzymes, essential to produce beer must (Bamforth, 1993). In this way, barley is transformed into malt.
  - Drying and Roasting: in a first drying phase the temperature is kept at 50-60 °C to reduce the humidity of the seed. Subsequently, the temperature is raised to around 80-90 °C depending on the type of malt desired. The type of malt used and the ways in which it is treated are fundamental for the quality of final product.

  The purposes of this phase are manifold: stop germination, reduce the humidity of green malt to increase its shelf life, make the malt crumblier, provide the desired aroma and colour to the malt (Bertinotti and Faraggi, 2015).
- Mashing: milled malt is mixed with hot water to extract cereal components (mainly starch) and to facilitate the enzymes activity. During this phase, starch is converted into fermentable sugar by enzyme action.
- Wort boiling: during this phase hops are added, giving the final flavour of the drink. Hops contain essential oils, which contribute to the hoppy, floral and spicy aromas in beer. Many of these the compounds are volatile and can therefore be lost by evaporation during boiling. To avoid this, the brewer can add some of the hops halfway or at the end through the boil. Furthermore, in this phase, the beer must start browning because of the reactions that occur between the reducing sugars and the primary amines (Hughes and Baxter, 2001).
- Fermentation: after boiling, the wort is cooled and then fermented. Beer fermentation is a key process to improve the quality of the final product. The various types in which beer is

distinguished also have to do with the characteristics of this process, as well as with the types of yeasts used, times, temperatures and processing methods. Two types of fermentation can be distinguished, top and bottom fermentation. In the first one, beer is fermented by yeast belonging to the *S. cerevisiae* species, operating at temperature between 12-23 °C and it manages a maturation process in about two weeks. This process includes English Ale, Trappist, Irish Stout and British Indian Pale Ale (IPA) beers.

Beer fermentation can also take place using different yeasts and temperature, by adding yeast from the *S. pastorianus* species to the must. These act at a temperature between 7-9 °C and require at least 5 or 6 weeks for complete maturation. Once fermentation is complete, the remains of the yeasts settle on the bottom. An example are the “lager” beers (Briggs et al., 2004).

- Maturation: after fermentation, the beer is racked into special tanks where it will be left from four to six weeks to mature. In this phase all its components are refined and stabilized, a sort of natural clarification takes place and the beer acquires its characteristic and definitive flavour. Most of the beers are then subjected to further filtration to eliminate any traces of yeasts and other components that would make the drink cloudy. At this point the beer is practically ready to be packaged and then consumed (Fix, 1989).

1.2 MEAD HISTORY AND PRODUCTION

The history of mead is long and interesting, since its discovery is probably fortuitous. As described by the Neolithic hypothesis (Rogers, 2014), men began drinking from beehives filled with rainwater that fell to the ground, so yeasts, supported by mild environmental temperatures, started spontaneous fermentations, generating this beverage. Mead is probably the first fermented beverage produced in history, even prior to wine and beer, thanks to the easy availability of the basic ingredients: honey and water. Archaeological evidence helps us to deduce that the production of mead dates to 7000 b.C. in northern China, where ceramic vessels containing traces of honey, rice and other fruits, were used for mead production. Moreover, in Celtic mythology, mead was considered a drink of the nobles and deities, providing immortality, knowledge and gift of poetry (Gupta and Sharma, 2009). Historically, mead was known for its healing and mystical properties, receiving legendary notoriety as the drink "ambrosia" or "nectar of the gods" in ancient Greece. In fact, the myth was passed down that the elixir composed of honey was the source of strength of the Norse god Odin, who drank it as a child from the udder of a goat (McCoy, 2016). Today, it is still popular among Northern European populations as a traditional drink. Although it is the most ancient fermented product of the world, mead is not quite easy to find in commerce, especially in the south of Italy, where the market of alcoholic beverages is mainly occupied by wine and beer.

As suggested by its etymology, the term "mead", derived from the Greek ὕδωρ, hýdor "water" and μέλι, méli "honey", refers to a traditional honey-based beverage containing 8 - 18% (v/v) ethanol, produced by the alcoholic fermentation of bee honey dissolved in an adequate amount of water (Pereira et al., 2014). Mead production is rapidly evolving with modern production techniques that follow the same path as craft beer production, albeit with different methodologies and fermentation times (American Mead Makers Association, 2017). The fermentation process lasts longer than other sugar matrix products because honey contain a high percentage of fructose and glucose and also small amounts of sucrose, resulting in high carbon-to-nitrogen ratio (Tysset and Rautlin de la Rox, 1974). In fact, fermentation often takes several months to convert all the sugar into alcohol, depending on the type of honey, the strain of yeast and the composition of the wort. Some problems can occur during this process, due to the inability of yeast strains to respond and adapt to stressful growth under the unfavourable conditions found in honey. As a result, complications such as lack of uniformity in the final product are likely caused by variability in the composition of the honey and honey-must, and the presence of refermentations due to altering yeasts or bacteria, which can increase volatile acidity and promote abnormal ester production, affecting the sensory qualities of the final product (Iglesias et al., 2014). Another key aspect that establishes the quality of the beverage is the colour of the honey, which depends on its potential alkalinity and ash content, as well as active antioxidant pigments such as carotenoids and flavonoids (Frankel et al., 1998). The colour shade of mead is derived from the type of used honey, ranging from straw yellow to brown. Studies conducted by Pereira et al. (2015) showed that the use of dark honey resulted in improved profile and fermentation conditions due to higher mineral and pH content.

The production of a good quality beverage depends on fermentation, botanical variety of the flora, yeast strain (mainly belonging to the *Saccharomyces* genus), pH and nutrients available to the yeasts, including nitrogen compounds (Ramalhosa et al., 2011).

Generally, the first step is the initial preparation of the mixture made of honey and water, in variable percentage according to the ethanol content to be reached at the end of the process of transformation of sugars by yeasts. The addition of nutrients is strictly required, to stimulate the fermentation vigour of yeast strain, in order to dominate the system and inhibit alternative microorganisms. Spices are added for flavouring and further distinguish the final product. Organic acids, like citric, malic or tartaric, are employed to regulate acidity and pH value.

Afterwards, to inactivate most of the spontaneous microflora of honey, pasteurization is usually performed. Subsequently the selected starter yeast is inoculated, beginning the fermentation phase, which can take up some months. The daily monitoring on its progress is crucial to avoid insidious effects on the final product and on the quality of mead itself (Starowicz et al., 2020). Therefore, once

fermentation is over, impurities are removed, then clarified, by centrifugation or by using clarifying agents, such as bentonite, isinglass, white egg, gelatin and casein, and filtered before bottling (Pereira et al., 2017).

Aging is the last phase of mead making, which is fundamental for the development of aromatic compounds that improve the organoleptic component. It can last from few months for light beverages, up to two years for meads with more alcohol which will need more time to fully develop (Ramalhosa et al., 2011).

1.3 FERMENTATIVE MICROORGANISMS: YEASTS AND BACTERIA

The central element of the transformation of a sugar must into an alcoholic beverage is fermentation, which is carried out by various microorganisms, like yeasts and bacteria.

1.3.1 *Saccharomyces* yeasts

Despite all changes made in beverage industry, the role of yeast remained the only constant factor. The modern brewing process was reached thanks to many changes made over time. Many beer styles have developed, all with their own unique character and flavour influenced by their production place (Protz, 1995; Glover, 2001). According to the flocculation behaviour and the yeast fermentation capacity, it is therefore possible to distinguish two types of fermentation: top fermenting (ale yeast) (Erten et al., 2014) and bottom fermenting (lager yeast). Ale yeast is genetically more diverse and ferments at higher temperatures (18-24 °C) while lager yeast is more conserved and ferments at lower temperatures (8-14 °C).

The genus *Saccharomyces*, belonging to the fungi kingdom, currently contains some of the most important species for the food and beverage industry. Nine species are reported in this genus: *S. cerevisiae*, *Saccharomyces paradoxus*, *Saccharomyces mikatae*, *Saccharomyces kudriavzevii, Saccharomyces arboricola, Saccharomyces eubayanus, Saccharomyces uvarum* and two hybrid species *S. pastorianus* and *Saccharomyces bayanus*.

The genus *Saccharomyces* is capable of interspecific hybridization and depending on the type of hybridization, genetic information is also shared in the form of chromosomal and mitochondrial DNA (Marinoni et al., 1999).

Interspecific hybridization occurs naturally between *S. cerevisiae* and *S. kudriavzevii* and between *S. cerevisiae* and *S. bayanus*. One of the possible causes could be an evolutionary adaptation to different environments (González et al., 2008).

Among these species, *S. cerevisiae* is considered as the agent of wine, bread, ale beer, and sake fermentations, *S. bayanus* in wine and cider fermentations, while *S. pastorianus* is involved for lager

beer fermentation (Rainieri et al., 2006). In particular, *S. pastorianus* strains come from a natural hybridization event that occurred between a *S. cerevisiae* and a non-*S. cerevisiae* strain. *S. cerevisiae* is a single-celled and osmophilic eukaryotic microorganism. This species can be found in a great variety of environments and matrices. Although its importance is often associated with the food sector, *S. cerevisiae* is the main player in the alcoholic fermentation of beverages, precisely because it can metabolize and convert sugar into ethanol (Cavalieri et al., 2003).

In a common culture medium for yeasts such as YPD (yeast extract-peptone-dextrose) *S. cerevisiae* is globular in shape and white in colour, while on WL Nutrient Agar medium, the colonies are large, light green with grey border, smooth, glossy surface, and buttery consistency with full margin. The optimal growth temperature is 30 °C, in optional anaerobic conditions (Dimmer et al., 2002).

The fermentation of beer conducted with *S. cerevisiae* is called "top fermentation", due to the position of the yeast on the surface of the wort. The fermentation temperature varies according to the single strain, ranging between 14 and 32 °C and it takes from 10 to 15 days for the fermentation to complete (Krescankova et al., 2015).

Regarding mead, *S. cerevisiae* yeasts of oenological derivation are usually employed. Several authors (Gomes et al., 2013; Pereira et al., 2015; Sottil et al., 2019) use or recommend white wine or champagne strains to drive alcoholic fermentation and for this reason, mead is also known as “honey wine” (Roldán et al., 2011). These commercial strains are technological screened for oenological parameters like fermentation vigour, power and purity, sedimentation capacity, aromatic compounds production (Bauer and Pretorius, 2000), however their use may not fit the honey-must composition, leading to stuck fermentations and negative sensorial characteristics (Pereira et al., 2009).

This microorganism is of fundamental relevance, not only because it determines the main characteristics of the fermented products through the synthesis of primary metabolites such as ethanol and carbon dioxide, but also because it contributes to define part of the qualitative characteristics, influencing above all the olfactory sensory aspect. *S. cerevisiae* is in fact, capable of generating hundreds of volatile compounds whose abundance, both qualitative and quantitative, is dependent on the used strain. These differences are implied by the natural metabolic biodiversity present within the species. In wine fermentation, for decades now, this natural variability has been exploited by offering on the market several selected *S. cerevisiae* strains having different metabolic and physiological characteristics, generally in the form of active dry yeast (Vaudano, 2014).

Another yeast of great importance for brewing industry is *S. pastorianus*. This lager-brewing yeast is now understood to be an interspecific hybrid involving *S. cerevisiae* and *S. eubayanus* (Libkind et al., 2011). The hybridisation event happened approximately 500-600 years ago and therefore *S. pastorianus* may be considered as a newly evolving species. It has unique genetic characteristics ideal

for fermenting sugars and therefore producing very tasty beers (Monerawela et al., 2017). This yeast is used in the production of lager-style beers, which fermentation requires very low temperatures compared to other industrial fermentation processes.

S. pastorianus operates at temperatures between 5-15 °C and unlike other, it doesn't give the final fruity or floral aromas and flavours (Gibson and Liti, 2015). The strains involved in lager fermentation, in addition to be cryotolerant and with good ability to ferment maltose and maltotriose (capacities inherited from the species of origin or due to hybridization), tend to settle after flocculation and to sink to the bottom of the fermentation vessel (Vidgren and Londesborough, 2011).

The fermentation process occurs in three main, chronologically distinct steps:

- Breathing: the yeast uses the oxygen dissolved in the must to actively start the fermentation and reproduction activity.
- Fermentation: yeast cells metabolize sugars transforming them into ethyl alcohol and carbon dioxide. The cells reproduce very quickly and the population doubles every 20 minutes, reaching levels of over 10 million yeasts in every millilitre of must.
- Sedimentation: when almost all the sugars have been consumed, the yeast starts to flocculate and settle on the bottom.

1.3.2 Non-*Saccharomyces* yeasts

Recently non-*Saccharomyces* yeasts have been studied for their peculiar metabolic and enzymatic pathways. The reasons for this interest are due to the criticisms that are increasingly driven by the fermentations guided by the massive initial inoculations of *S. cerevisiae* commercial strains. According to several authors, the exclusive use of *S. cerevisiae* starter yeast would lead to a flattening of the sensory characteristics of the finished products, as regards the olfactory sensory aspect. During spontaneous fermentation, development and succession of different species and strains could give greater complexity and distinctive sensorial characteristics to the final product (Vaudano et al., 2014). Yeasts responsible for spontaneous fermentation, the so-called non-*Saccharomyces* yeasts, are also called "indigenous" or "wild" yeasts to distinguish them from the added exogenous yeast cultures (Varela, 2016). The metabolism of these yeasts is responsible for the formation of several hundred active-aromatic compounds which give to fermented drinks their characteristic aroma and flavour.

Indigenous yeasts are generally unable to complete alcoholic fermentation due their low alcohol tolerance and for excessive production of secondary compounds. For this reason, they are often used in co-inoculation with *S. cerevisiae* (Vaudano et al., 2014).

Several non-*Saccharomyces* yeast species are found during spontaneous fermentations of certain beer styles (Belgian Lambic beer and American coolship ales), including *Meyerozyma guilliermondii*, *Debaryomyces spp.*, *Pichia spp.*, *Wickerhamomyces anomalus*, *Brettanomyces anomalus*,

Brettanomyces custersii, Brettanomyces bruxellensis, *Candida krusei, Cryptococcus keutzingii* and *Rhodotorula mucilaginosa* (Bokulich and Mills, 2012; Spitaels et al., 2014).

The Table 1 shows a list of the most common non-*Saccharomyces* yeast species found during spontaneous fermentation in different alcoholic beverages.

Table 1. List of the most common non-*Saccharomyces* yeast species found during spontaneous fermentation in different alcoholic beverage (Varela, 2016).

Species	Fermentation source
Brettanomyces anomalus	Beer
Brettanomyces bruxellensis	Wine, beer, tequila
Candida krusei	Beer
Hanseniaspora guilliermondii	Wine, tequila, cachaça
Hanseniaspora uvarum	Wine
Issatchenkia occidentalis	Tequila
Issatchenkia orientalis	Wine
Lachancea thermotolerans	Wine
Metschnikowia pulcherrima	Wine
Pichia caribbica	Mezcal, cachaça
Pichia fermentans	Wine, beer, cachaça
Rhodotorula mucilaginosa	Wine, beer, mezcal
Starmerella bacillaris	Wine
Wickerhamomyces anomalus	Wine, beer
Zygosaccharomyces florentinus	Wine, beer

1.3.3. Lactic Acid Bacteria

Lactic acid bacteria (LAB) are microorganisms that mainly produce lactic acid from the fermentation of sugars. These bacteria are essentially ubiquitous and normally found in food. They can have a coccyx or rod shape, they are Gram positive and catalase negative (Pfeiler and Klaenhammer, 2007). LAB have non-motile cells and do not produce spores; they are anaerobic or microaerophilic, therefore, they grow well in total or partial absence of oxygen. This taxon includes a variety of genus including some of industrial relevance such as *Lactococcus, Enterococcus, Oenococcus, Pediococcus*, *Streptococcus, Leuconostoc* and *Lactiplantibacillus* (Makarova et al., 2006). LAB have an exclusively fermentative metabolism even in the presence of oxygen. Many are anaerobic because of the lack of catalase which prevents them from degrading toxic forms of oxygen such as hydrogen peroxide, which forms during oxidative metabolism. From a nutritional point of view, they have complex nutritional requirements: they need a carbon source (such as glucose), vitamins, mineral elements (such as manganese and magnesium) and a source of nitrogen (Schroeter et al., 2009).

Thanks to the presence of amino acids, vitamins and fermentable compounds such as residual sugars, beer wort represents a nutrient medium that can allow LAB development. However, only a small part, compared to the multitude of those present in the food sector, can develop in beer with positive value due to the highly selective conditions. For example, the hop iso α-acids, exert antibacterial effects,

providing an additional layer of defence against bacteria, which may inadvertently spoil into finished beer products. Nevertheless, it has been reported that approximately 60-70% of beer spoilage incidents are caused by LAB. The main microbial beer deterioration has been shown to be caused by *Levilactobacillus brevis*, *Fructilactobacillus lindneri*, *Secundilactobacillus paracollinoides* and *Pediococcus damnosus* species (Suzuki et al., 2008).
The role of LAB in beer production has recently been subverted, thanks to an increase in popularity of so-called sour beers (Bossaert et al., 2019). This beer style is generally obtained by the combined use of bacteria and yeast strains. The most important genera involved in sour beers are *Lactobacillus* and *Pediococcus*, mainly used in pre-fermentative phase. Prior to yeast inoculum, selected LAB cultures are pitched in unhopped wort, reaching the desired level of acidification and finally inactivated thanks to boiling phase (Tonsmeire, 2014).

1.4. YEAST NUTRITION

In natural environments, yeasts can utilize a wide range of nitrogen-containing compounds. The assimilation of these compounds can occur in different ways and degrees, based on the nature of the nitrogen compound and the species using it, generally optimizing their growth and metabolic activity (Barbosa et al., 2012). The influence of carbon and nitrogen supply on yeast fermentative aroma is well known, since several studies focused on this aspect, especially regarding wine production. The main fruity or floral aroma-active compounds in wine are attributable to the work of yeast during AF, and their synthesis can be strongly affected by winemaking practices. Many production aid tools are available in the oenological market, such as enzymes, clay minerals, organic acids, antioxidants and yeast nutrients, which can promote a smooth course of alcoholic fermentation and, in general, the quality of the final wine (Alfonzo et al., 2021; Claus et al., 2018; Kemp et al., 2015). Depending on the composition of the matrix to be fermented, the yeast will encounter different working conditions, being fermentation environment variable in terms of pH, acidity, available sugars, assimilable nitrogen, vitamins, mineral salts, presence of inhibiting factors.
Compared to wine must, honey-must lacks in Yeast Assimilable Nitrogen (YAN), which could be four time lower than the optimal fermentation threshold of 150 mg/L (Ribéreau-Gayon et al., 2006), depending on the water dilution. This fact can halt or slow alcoholic fermentation, leading to the synthesis of off-odour, like sulfur derivatives. For this reason, a common practice is the use of diammonium phosphate (DAP). Almeida et al. (2020) highlighted that the use of supplementation of honey must with DAP or ammonium sulphate led to a higher sugar consumption and higher alcoholic content. Roldán et al. (2011) successfully used pollen, the most important source of proteins, lipids, mineral and vitamins for bee survival, as fermentative activator to improve fermentation course and

final characteristics of mead. The presence of antifungal components in honey is another element that makes the adaptation of yeast strains in these matrices difficult (Gomes et al., 2010).

In brewing, recently, particular attention is being paid to the fermentation of the so-called "High Gravity" beers. Besides being a high alcohol-by-volume type of beer, HG worts can be used to increase the production efficiency of the brewery. This method is mainly used for lager beer and almost always involves the addition of sugary syrups in a standard wort, thus unbalancing the composition of the wort in disadvantage of nitrogen (Lei et al., 2012). Several authors focused on this aspect. Li et al. (2019) successfully applied protein hydrolysates from defatted walnut meal as a nitrogen source in high gravity wort, improving yeast growth and viability, glycogen and trehalose accumulation, as well as an increase of higher alcohols and esters, obtaining a better balance taste of final beer. Yang et al. (2018) used the key amino acids for brewing yeast Lys and Leu, in the form of peptide and mixture of simple amino acids, to improve yeast fermentation performance on a very High Gravity wort of 24 °P. Ciosek et al. (2020) evaluated the supplementation with magnesium and zinc ions for sour beer production, where LABs are employed for lactic acid production in a mixed fermentation with yeasts, demonstrating that ions supplementation could influence positively or negatively the LAB strain growth, pH decrease, lactic acid production and volatile compounds concentrations. Ribeiro-Filho et al. (2021), studied the effect of nine different experimental treatments (ammonia nitrogen, inorganic phosphate, potassium, magnesium, copper, zinc, iron, manganese and a composite mixture) on three different *Saccharomyces* strains, highlighting significant changes in terms of aroma compounds among treatments.

In summary, especially when fermentation conditions of musts are particularly complex, such as very high-gravity and sour beers, it is necessary to pay attention to the nutrition of microorganisms, to guarantee the absence of faults due to stuck fermentation or fermentation in presence of nutritional stress.

1.5. FERMENTATIVE CULTURES INOCULUM STRATEGY

The increasing demand for breakthrough and unique beverages enabled numerous opportunities and strategies to create novel fermented products, like beers or meads (Habschied et al 2020; Iglesias et al., 2014). The great potential of this market segment is confirmed by the constant increase in production units of breweries and meaderies in many countries, encouraged by the consumers willingness to always try new products. To achieve this goal, the strategy of increasing microbial and fermentative complexity seems promising. Based on the established success of Belgian Lambic and in general of mixed fermentation beers, several developments are going on in this direction.

In wine sector, there are numerous studies that have shown that the use of multiple yeast strains, used simultaneously or sequentially, is a widespread and well-established strategy to increase the complexity and quality value of wine production. This subject has in fact been under investigation in Italy for several years, as demonstrated by the study conducted by Zironi et al. (1993).

Numerous species, including *Torulaspora delbrueckii* (Simonin et al., 2018), *Metschnikowia pulcherrima* (Canonico et al., 2019; Morata et al., 2019), *Kluyveromyces marxianus* (Barone et al., 2021), *Candida zemplinina* (Di Maio et al., 2013), *Pichia kluyveri* (Hu et al., 2021), *Lachancea thermotolerans* (Vaquero et al., 2021) have been employed in oenology in the last decade to pursue several objectives, like microbial acidification, pre-fermentation bio-protection, increased glycerol, reduced ethanol and in general improve the overall quality of wines.

In brewing, with a few years delay respect to the wine sector, the same practices are spreading to obtain innovative productions with non-conventional fermentations, using non-*Saccharomyces* strains. The main reasons for using non-conventional yeasts in brewing include bio-flavouring, acidification, development of enzymes that release aromatic precursors, production of low calorie and alcohol beers, production of probiotic or enriched beers (Holt et al., 2018; Puligundla et al., 2021). The use of more than one species, requires an in-depth study on interactions between different microorganisms, considering the compatibility and competition between the strains involved in fermentation. The use of different microorganisms can be done in different phases.

LABs are often used alone in the kettle souring phase, by pitching the selected bacterial species (*Lactiplatibacilluss* spp. or *Pediococcus* spp.) in absence of hops, before boiling. The wort is then left to acidify until a target pH is reached, which usually occurs in 24-48 hours. Subsequently the brewing proceeds in the classic way, with boiling, hopping, cooling, yeast inoculation and subsequent start of primary fermentation.

Among the advantages of a temporal separation of the inoculation of the two species, with the technique of sequential inoculation, there is a greater control of the conditions of temperature, oxygenation and nutrition for each culture individually, allowing a greater production replicability. Peyer et al. (2017) confirmed that the use of separate souring before boiling could be the best method to obtain an increase in acidity, with minimal risks of organoleptic failures.

To enhance bio-flavouring, Holt et al. (2018) screened 17 non-conventional yeast species, inoculating them 48 h before the use of a commercial strain of *S. cerevisiae*.

Canonico et al. (2016) used *T. delbrueckii* DiSVA 254 strain in co-inoculum with commercial US-05 *S. cerevisiae* starter strain at different ratio (1:1, 10:1, 20:1) and demonstrated that the non-*Saccharomyces* strain affected the analytical and aromatic beer profile when the inoculation ratio was

above 10:1 (*T. delbrueckii*/*S. cerevisiae*). Under these mixed fermentation conditions, a higher YAN consumption was registered, probably related to aromatic compounds synthesis.

The presence of more than one inoculated strain from different species can be improved by modulating parameters like temperature. In any case, the presence of a secondary strain affects the growth of the principal one, as reported by Gobbi et al. (2013).

In mead, very limited papers have been published to date on the combined use of multiple yeasts. Li and Sun (2019) employed separately two different commercial strains of *L. thermotolerans* and *T. delbrueckii*, in combination with the commercial *S. cerevisiae* oenological strain EC1118, sequentially inoculated after 48 hours of the non-*Saccharomyces* strain pitching in vitex honey must. *L. thermotolerans/S. cerevisiae* stood out for the highest honey aroma, taste quality, and overall impression, *T. delbrueckii*/*S. cerevisiae* improved the fruity aroma while the use of single culture EC1118 resulted in unbalanced mead, with pronounced notes of "soapy", "candlewax", "oily" and "fatty" descriptors.

Lopes et al. (2020) instead inoculated *M. caribbica* and *S. cerevisiae* simultaneously in mead must, with the non-*Saccharomyces* inoculated in a higher concentration than *S. cerevisiae* to increase its prevalence along with *S. cerevisiae.*

In conclusion, as evidenced by the above studies, there does not seem to be one mode of culture inoculation that guarantees better results than others absolutely. Rather, the choice of a sequential inoculum versus a co-inoculum, must be evaluated case by case, according to the type of matrix to be fermented, the conditions of pH, sugars, YAN, temperatures and compatibility of used strains.

1.6. REFERENCES

Alfonzo, A., Prestianni, R., Gaglio, R., Matraxia, M., Maggio, A., Naselli, V., …, Francesca, N. (2021). Effects of different yeast strains, nutrients and glutathione-rich inactivated yeast addition on the aroma characteristics of Catarratto wines. Int. J. of Food Microbiol. 360, 109325.

Almeida, E.L.M.D., Moreira e Silva, G., Vassalli, I.D.A., Silva, M.S., Santana, W.C., Silva, P.H.A.D., Eller, M.R. (2020). Effects of nitrogen supplementation on *Saccharomyces cerevisiae* JP14 fermentation for mead production. Food Sci. Technol. 40, 336-343.

American Mead Makers Association. (2017). What's the Buzz? 2017 Mead Industry Report. American Mead Makers Association: Seattle, WA, USA.

Arnold, J.P. (1911). Origin and History of Beer and Brewing.

Bamforth, C.W., Barclay, A.H.P., 1993. Malting technology and the uses of malt. Barley: chemistry and technology, 297-354.

Barone, E., Ponticello, G., Giaramida, P., Squadrito, M., Fasciana, T., Gandolfo, V., …, Oliva, D. (2021). Use of *Kluyveromyces marxianus* to Increase Free Monoterpenes and Aliphatic Esters in White Wines. Fermentation, 7(2), 79.

Bauer, F.F., Pretorius, I.S. (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine- a review. South African J. Enol. and Vitic. 21, 27–51.

Baxter, E.D., Hughes, P.S. (2001). Beer: Quality, safety and nutritional aspects. Royal Society of Chemistry.

Bertinotti, D., Faraggi, M., Mobi, M.B.I. (2015). La tua birra fatta in casa. Edizioni LSWR.

Bokulich, N.A., Mills, D.A. (2012). Microbial terroir of wine: deep insight into site-specific winery and vineyard microbial communities. Am. J. Enol. Viticult. 63:435a–435a.

Bossaert, S., Crauwels, S., De Rouck, G., Lievens, B. (2019). The power of sour-a review: old traditions, new opportunities. Brew. Sci. 72(3-4), 78-88.

Briggs, D. E., Brookes, P. A., Boulton, C. A., Stevens, R. (2004). Brewing: science and practice (Vol. 108). Woodhead Publishing.

Canonico, L., Agarbati, A., Comitini, F., Ciani, M. (2016). *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. Food microbiol. 56, 45-51.

Cavalieri, D., McGovern, P. E., Hartl, D. L., Mortimer, R., and Polsinelli, M. (2003). Evidence for *S. cerevisiae* fermentation in ancient wine. J. Mol. Evol. 57(1), S226-S232.

Ciosek, A., Fulara, K., Hrabia, O., Satora, P., Poreda, A. (2020). Chemical Composition of Sour Beer Resulting from Supplementation the Fermentation Medium with Magnesium and Zinc Ions. Biomolecules, 10(12), 1599.

Claus, H., Mojsov, K. (2018). Enzymes for wine fermentation: Current and perspective applications. *Fermentation*, *4*(3), 52.

Di Maio, S., Genna, G., Gandolfo, V., Amore, G., Ciaccio, M., Oliva, D. (2012). Presence of *Candida zemplinina* in Sicilian musts and selection of a strain for wine mixed fermentations. South African J. Enol. Vitic. 33(1), 80-87.

Dimmer, K. S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W., Westermann, B. (2002). Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*. Molecular biology of the cell, 13(3), 847-853.

Erten, H., Ağirman, B., Gündüz, C.P.B., Çarşanba, E., Sert, S., Bircan, S., Tangüler, H. (2014). Importance of yeasts and lactic acid bacteria in food processing. In Food Processing: Strategies for Quality Assessment (pp. 351-378). Springer, New York, NY.

Fix, G. (1989). Principles of Brewing Science, Brewers. Boulder, Colorado: Brewers. Chapman-Hall, Ltd.

Frankel, S., Robinson, G.E., Berenbaum, M.R. (1998). Antioxidant capacity and correlated characteristics of 14 unifloral honeys. J. Apic. Res. 37(1), 27-31.

Giardini, L., Baldoni, R. (2000). Coltivazioni erbacee. Coltivazioni erbacee, 1-396.

Gibson, B., Liti, G. (2015). *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. Yeast, 32(1), 17-27.

Glover, B. (2001). The World Encyclopedia of Beer. Lorenz Books, Anness Publishing limited, New York.

Gobbi, M., Comitini, F., Domizio, P., Romani, C., Lencioni, L., Mannazzu, I., Ciani, M. (2013). *Lachancea thermotolerans* and *Saccharomyces cerevisiae* in simultaneous and sequential co-fermentation: a strategy to enhance acidity and improve the overall quality of wine. Food microbiology, 33(2), 271-281.

Gomes, S., Leandro, L. G., Moreira, L., Rodrigues, P., Estevinho, L. (2010). Physicochemical, microbiological and antimicrobial properties of commercial honeys from Portugal. Food and Chemical Toxicology, 48, 544–548.

Gomes, T.; Barradas, C.; Dias, T.; Verdial, J.; Morais, J.S.; Ramalhosa, E.; Estevinho, L.M. (2013). Optimization of mead production using response surface methodology. Food Chem. Toxicol., 59, 680–686.

González, S.S., Barrio, E., Querol, A. (2008). Molecular characterization of new natural hybrids of *Saccharomyces cerevisiae* and *S. kudriavzevii* in brewing. Appl. Environ. Microbiol. 74(8), 2314-2320.

Gupta, J.K., Sharma, R. (2009). Production technology and quality characteristics of mead and fruit-honey wines: A review. Indian J. Nat. Prod. Resour. 8, 345-355.

Habschied, K., Živković, A., Krstanović, V., Mastanjević, K. (2020). Functional beer - A review on possibilities. Beverages, 6(3), 51.

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K.J., Thevelein, J.M. (2018). Bioflavoring by non-conventional yeasts in sequential beer fermentations. Food Microbiol. 72, 55-66.

Hornsey, J.S., 2003. A History of Beer and Brewing. Royal Society of Chemistry.

Hu, K., Zhao, H., Edwards, N., Peyer, L., Tao, Y., Arneborg, N. (2022). The effects of cell-cell contact between *Pichia kluyveri* and *Saccharomyces cerevisiae* on amino acids and volatiles in mixed culture alcoholic fermentations. Food Microbiol. 103, 103960.

Iglesias, A., Pascoal, A., Choupina, A.B., Carvalho, C.A., Feás, X., Estevinho, L.M. (2014). Developments in the fermentation process and quality improvement strategies for mead production. Molecules, 19(8), 12577-12590.

Katz, S.H., Maytag, F., 1991. Brewing an ancient beer. Archaeology, 44(4), 24- 27.

Kemp, B., Alexandre, H., Robillard, B., Marchal, R. (2015). Effect of production phase on bottle-fermented sparkling wine quality. J. Agricultural and Food Chemistry, 63(1), 19-38.

Krescanková, K., Kopecká, J., Němec, M., Matoulková, D. (2015). Characterization of technologically utilized *Saccharomyces yeast*. KVASNÝ PRŮMYSL, 61(6), 174-185.

Legras, J.L., Cornuet, J.M., Karst, F. (2007). Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. In 26. International Specialised Symposium on Yeasts (pp. 21-p).

Lei, H., Zhao, H., Yu, Z., Zhao, M. (2012). Effects of wort gravity and nitrogen level on fermentation performance of brewer's yeast and the formation of flavor volatiles. Appl. Biochem. Biotechnol. 166(6), 1562–1574.

Li, R., Sun, Y. (2019). Effects of honey variety and non-*Saccharomyces cerevisiae* on the flavor volatiles of mead. J. Am. Soc. Brew. Chem. 77(1), 40-53.

Libkind, D., Hittinger, C.T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P. Sampaio, J. P. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc. Natl. Acad. Sci. 108(35), 14539-14544.

Lopes, A.C.A., Costa, R., Andrade, R.P., Lima, L.M.Z., Santiago, W.D., das Graças Cardoso, M., Duarte, W.F. (2020). Impact of *Saccharomyces cerevisiae* single inoculum and mixed inoculum with *Meyerozyma caribbica* on the quality of mead. European Food Research and Technology, 246(11), 2175-2185.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A. Pavlova, …, Shakhova, V. (2006). Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. 103(42), 15611-15616.

Marinoni, G., Manuel, M., Petersen, R. F., Hvidtfeldt, J., Sulo, P., Piskur, J. (1999). Horizontal transfer of genetic material among *Saccharomyces yeasts*. J. Bacteriol., 181 (1999), pp. 6488-6496.

McCoy, D. (2016). The Viking spirit: An introduction to Norse mythology and religion. CreateSpace Independent Publishing Platform.

Meussdoerffer, F.G. (2009). A comprehensive history of beer brewing. Handbook of brewing: Processes, technology, markets, 1-42.

Monerawela, C., Bond, U. (2017). Brewing up a storm: The genomes of lager yeasts and how they evolved. Biotechnol. Adv., 35(4), 512-519.

Morata, A., Loira, I., Escott, C., del Fresno, J.M., Bañuelos, M.A., Suárez-Lepe, J.A. (2019). Applications of *Metschnikowia pulcherrima* in wine biotechnology. Fermentation, 5(3), 63.

Pereira, A.P., Dias, T., Andrade, J., Ramalhosa, E., Estevinho, L.M. (2009). Mead production: Selection and characterization assays of *Saccharomyces cerevisiae* strains. Food Chem. Toxicol. 47(8), 2057-2063.

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A. (2014). Effect of *Saccharomyces cerevisiae* cells immobilisation on mead production. LWT-Food Sci. Technol. 56(1), 21-30.

Pereira, A.P., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A. (2015). Improvement of mead fermentation by honey-must supplementation. J. Inst. Brew. 121(3), 405-410.

Pereira, A.P., Oliveira, J.M., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A. (2017). Mead and other fermented beverages. In: Current Developments in Biotechnology and Bioengineering (pp. 407-434). Elsevier.

Peyer, L.C., Zarnkow, M., Jacob, F., De Schutter, D.P., Arendt, E.K. (2017). Sour brewing: impact of Lactobacillus amylovorus FST2. 11 on technological and quality attributes of acid beers. J. Am. Soc. Brew. Chem. 75(3), 207-216.

Pfeiler, E.A., Klaenhammer, T.R., 2007. The genomics of lactic acid bacteria. Trends Microbiol. 15(12), 546-553.

Protz, R., 1995. The ultimate encyclopedia of beer: the definitive guide to the world's great brews. Carlton.

Puligundla, P., Smogrovicova, D., Mok, C. (2021). Recent innovations in the production of selected specialty (non-traditional) beers. Folia Microbiologica, 1-17.

Rainieri, S., Kodama, Y., Kaneko, Y., Mikata, K., Nakao, Y., Ashikari, T. (2006). Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. Appl. Environ. Microbiol. 72(6), 3968-3974.

Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T., Estevinho, L.M. (2011). Mead production: Tradition versus modernity. In Advances in food and nutrition research (Vol. 63, pp. 101-118). Academic Press.

Ribeiro-Filho, N., Linforth, R., Powell, C.D., Fisk, I.D. (2021). Influence of essential inorganic elements on flavour formation during yeast fermentation. Food Chem. 361, 130025.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A. (2006). Handbook of enology, Volume 1: The microbiology of wine and vinifications (Vol. 1). John Wiley & Sons.

Rogers, A. (2014). Proof: the science of booze. Houghton Mifflin Harcourt.

Roldán, A., Van Muiswinkel, G. C. J., Lasanta, C., Palacios, V., Caro, I. (2011). Influence of pollen addition on mead elaboration: Physicochemical and sensory characteristics. Food Chem. 126(2), 574-582.

Schroeter, J., Klaenhammer, T. (2009). Genomics of lactic acid bacteria. FEMS Microbiol. Lett. 292(1), 1-6.

Simonin, S., Alexandre, H., Nikolantonaki, M., Coelho, C., Tourdot-Maréchal, R. (2018). Inoculation of *Torulaspora delbrueckii* as a bio-protection agent in winemaking. Food Res. Int. 107, 451-461.

Sottil, C., Salor-Torregrosa, J.M., Moreno-Garcia, J., Peinado, J., Mauricio, J.C., Moreno, J., Garcia-Martinez, T. (2019). Using *Torulaspora delbrueckii*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus* wine yeasts as starter cultures for fermentation and quality improvement of mead. Eur. Food Res. Technol. 245(12), 2705-2714.

Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.M., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2014 The microbial diversity of traditional spontaneously fermented lambic beer. PLoS One 9.

Starowicz, M., Granvogl, M. (2020). Trends in food science & technology an overview of mead production and the physicochemical, toxicological, and sensory characteristics of mead with a special emphasis on flavor. Trends Food Sci. Technol. 106, 402-416.

Suzuki, K., Asano, S., Iijima, K., Kuriyama, H., Kitagawa, Y. (2008). Development of detection medium for hard-to-culture beer-spoilage lactic acid bacteria. J. Appl. Microbiol. 104(5), 1458-1470.

Tonsmeire, M. (2014). American sour beer: innovative techniques for mixed fermentations. Brewers Publications.

Tysset, C., Rautlin de la Roy Y., (1974). Study of osmophilic yeasts that ferment honeys produced in France. Bull. Assoc. Diplomes Microbiol. Fac. Pharm. Nancy, 134, 1-26.

Vaquero, C., Izquierdo-Cañas, P.M., Mena-Morales, A., Marchante-Cuevas, L., Heras, J.M., Morata, A. (2021). Use of *Lachancea thermotolerans* for Biological vs. Chemical Acidification at Pilot-Scale in White Wines from Warm Areas. Fermentation, *7*(3), 193.

Varela, C. (2016). The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. Appl. Microbiol. Biotechnol. 100(23), 9861-9874.

Vaudano, E., Bertolone, E., Petrozziello, M., and Garcia-Moruno, E., 2014. L'utilizzo dei lieviti non-*Saccharomyces* in fermentazione. L'Enologo, (1), 83-89.

Vidgren, V., Londesborough, J., 2011. 125th anniversary review: yeast flocculation and sedimentation in brewing. J. Inst. Brew. 117(4), 475-487.

Yang, H., Zong, X., Cui, C., Mu, L., Zhao, H. (2018). Peptide (Lys–Leu) and amino acids (Lys and Leu) supplementations improve physiological activity and fermentation performance of brewer's yeast during very high-gravity (VHG) wort fermentation. Biotechnol. Appl. Biochem. *65*(4), 630-638.

Zironi, R., Romano, P., Suzzi, G., Battistuta, F., Comi, G. (1993). Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. Biotechnol. Lett. 15, 235–238.

CHAPTER 2

Non-conventional yeasts from fermented honey by-products: focus on Hanseniaspora uvarum strains for craft beer production

ABSTRACT

The increasing interest in novel beer productions focused on non-*Saccharomyces* yeasts in order to pursue their potential in generating groundbreaking sensory profiles. Traditional fermented beverages represent an important source of yeast strains which could express interesting features during brewing. A total of 404 yeasts were isolated from fermented honey by-products and identified as *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *Hanseniaspora uvarum.* Five *H. uvarum* strains were screened for their brewing capability. Interestingly, *H. uvarum* strains showed growth in presence of ethanol and hop and a more rapid growth than the control strain *S. cerevisiae* US-05. Even though all strains showed a very low fermentation power, their concentrations ranged between 7-8 log cycles during fermentation. The statistical analyses showed significant differences among the strains and underlined the ability of YGA2 and YGA34 to grow rapidly in presence of ethanol and hop. The strain YGA34 showed the best technological properties and was selected for beer production. Its presence in mixed- and sequential-culture fermentations with US-05 did not influence attenuation and ethanol concentration but had a significant impact on glycerol and acetic acid concentrations, with a higher sensory complexity and intensity, representing promising co-starters during craft beer production.

2.1. INTRODUCTION

Beer is one of the oldest and most widely consumed alcoholic drink in the world. The first record of the production of a beer-like drink is reported by the Sumerians, about 6,000 years ago (Katz and Maytag, 1991). The modern brewing process is the result of continuous adjustments made over time. Many kinds of beer were born and established worldwide, each with its own distinctive feature, often linked to the production area (Glover, 2001; Protz, 1995). In recent years, the manufacture of craft beers included local cereal varieties as well as fruits in order to provide territorial ties.

Together with *Saccharomyces* other yeasts such as *Candida, Brettanomyces/Dekkera, Debaryomyces, Rhodotorula, Cryptococcus* and *Pichia* are detected in traditional beer styles, including Lambic, Gueuze, Belgian red-brown acidic ale and American coolship ale, subjected to spontaneous fermentations (Bokulich et al., 2012; Snauwaert et al., 2016; Spitaels et al., 2014). Although the interest on conventional *Saccharomyces* strains isolated from non-brewing matrices, like sourdough (Catallo et al., 2020; Marongiu et al., 2015), or distilleries (Araújo et al., 2018) is still strong, the application of non-*Saccharomyces* yeasts in beer is increasing. This phenomenon depends on their enzymatic activities that influence the aromatic profiles of the beers, mainly by the modulation of esters influenced by the activity of esterase enzymes (Pires et al., 2014) or through the shaping of terpenes and higher alcohols (Lappe-Oliveras et al., 2008). Furthermore, the reduction of

calories, the production of low levels of alcohol, the souring, the production of lactic acid and the generation of glycerol during fermentation by non-*Saccharomyces* yeasts might differentiate the existing beers or even create new styles (Cubillos et al., 2019; Michel et al., 2016). Due to specific substrate assimilation patterns, non-*Saccharomyces* yeasts produce several compounds (i.e., glycerol, lactic acid, acetaldehyde and ethyl acetate) useful to the productions of typical and distinguishable beers (Ciani and Comitini, 2011; Cordero-Bueso et al., 2013; Gonzlez et al., 2013; Johnson, 2013) or even assimilate sugars like maltose or maltotriose, which are not commonly assimilated by non-domesticated yeasts (Nikulin et al., 2020).

As reported by Sannino et al. (2019), great attention has been paid to some non-*Saccharomyces* yeasts, mainly Ascomycota genera, namely, *Pichia*, *Saccharomycodes*, *Zygosaccharomyces*, *Hanseniaspora* and *Torulaspora* for their possible exploitation as starter cultures in brewing. Also the common wine spoilage yeasts *Dekkera/Brettanomyces* have been exploited for their brewing potential (Lentz et al., 2014). They generally present low fermentation yields and are more sensitivity to ethanol but, in some cases, they can improve other characteristics such as texture and integration of aroma for the creation of a final peculiar bouquet. Actually, the application of non-*Saccharomyces* strains characterized by an intense production of enzymes, mainly hydrolases, such as glucosidase capable of releasing aroma precursors or aroma-active substances, but unable to perform the alcoholic fermentation (AF), represents an encouraging strategy for bio-flavouring fermented beverages (Rodriguez et al., 2007, 2010; Ruiz et al., 2018; Sadineni et al., 2012). Recent studies showed that *Hanseniaspora* genus can play several roles during a brewing process. Bellut et al. (2018) evaluated two different strains of *Hanseniaspora vineae* and *Hanseniaspora valbyensis* during alcohol-free beer production: both strains showed high viability rates, but only marginal differences, in terms of volatile profiles, with the control production carried out with *Saccharomyces cerevisiae* were registered. Larroque et al. (2021) successfully applied a strain of *H. vineae*, previously used in co-fermentation of wine, for the improvement of the fruity organoleptic characteristics of beer.

From this perspective, non-*Saccharomyces* strains are usually used in co-fermentation because ethanol production is performed by *Saccharomyces* species (Cubillos et al., 2019). According to Cubillos et al. (2019), the yeast diversity of several traditional fermented beverages is still uninvestigated and they represent potential ecological niches to isolate novel strains for brewery technologies.

With regards to uninvestigated traditional alcoholic beverages, several fermented alcoholic products that are typical of restricted geographical areas are produced in Sicily. Often, these beverages are expression of the local culture, history and folklore. Among these, "Spiritu Re' Fascitrari" (SRF) is a distillate liqueur produced from honey by-products at Sortino (Syracuse province, Sicily region,

Italy). The term "fascitrari" comes from the ancient hives made with stems of the plant *Ferula communis* called "fascitro" in the local dialect (Ajovalasit and Columba, 1998).
SRF is obtained from the recovery process of beeswax from opercula and residual substances from honey production. Residual water from wax recovery process, after fermentation, is distilled and blended with a decoction of honey and various aromas. Gaglio et al. (2017) described the main yeast species active during SRF spontaneous AF as belonging to the species *Zygosaccharomyces bailii, Zygosaccharomyces rouxii, S. cerevisiae, Pichia kudriavzevii, Wickerhamomyces anomalus* and *Lachancea fermentati*. SRF matrices certainly represent a microbial source of starter strains to be used in fermented beverage industries (Varela et al., 2016). Sinacori et al. (2014) who investigated the microbial ecology associated to honey of different geographical and botanical origins, highlighted the presence of various yeast species of interest as beer starter cultures.
For this reason, the present study aimed to: (i), isolate and identify yeasts from fermented honey by-products (FHP); (ii), characterize the yeasts strains for their main brewing traits; (iii), select non-*Saccharomyces* yeasts as potential co-starter for fermenting wort and/or green beer; (iv), evaluate the effect of *H. uvarum* inoculum during beer fermentation and sensory quality of bottled products.

2.2. MATERIALS AND METHODS

2.2.1. Sampling and microbiological analysis of fermented honey by-products

A total of 12 samples of FHP were collected during four independent productions of SRF distillate, with three replicates for each sampling. In details, the watery solution of pressed watery decoction was spontaneously fermented at the honey company "G. Pagliaro" located in Sortino (Siracusa, Sicily, Italy) and the samples were collected at the end of the AF, in order to isolate yeasts with higher ethanol resistance. All samples were transferred into sterile plastic bags (BagLight® 400 Multilayer® bags, Interscience, Saint Nom, France) and transported with a portable fridge to the laboratory of Agricultural Microbiology, SAAF (Agricultural, Food and Forest Science) Department - University of Palermo (Italy).
The microbiological analysis was performed to investigate the presence of yeasts associated to FHP following the same procedures published by Gaglio et al. (2017) to count and isolate total osmophilic yeasts, total osmotolerant yeasts and total yeasts, during SRF production phases. Briefly, samples were homogenized in glucose solution to avoid cell damages. Liquid samples were subjected to decimal serial dilution in 30% (*w/v*) glucose solution. Total yeast counts were performed after sample dilution in peptone water solution (Sinacori et al., 2014). Total osmophilic yeasts were cultivated on De Walley Agar (DWA), total osmotolerant yeasts on tryptone glucose yeast extract agar (TGY), total yeasts were on Wallerstein laboratory (WL) nutrient agar, while non-*Saccharomyces* yeasts on

Lysine Agar (LA). When no colony developed, the following enrichment procedure was applied: 50 g or 50 mL of each sample were added to 50 mL of yeast extract peptone dextrose (YPD) and incubated statically for 5 and 10 d at 25 °C. All media were purchased from Oxoid (Basingstoke, Hampshire, UK). Microbiological counts were performed in triplicate.

2.2.2. Isolation and genotypical identification of yeasts

After growth, all isolates were picked up from each of the four media used for microbial count and purified by successive sub-culturing onto Malt Extract Agar (MEA). Yeast isolates were identified by molecular methods. DNA was extracted by cell lysis using the InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. To perform a first discrimination of the yeasts, all isolates were analysed by restriction fragment length polymorphism (RFLP) of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. DNA amplification occurred with the primer pair ITS1/ITS4 according to Esteve-Zarzoso et al. (1999). The generated amplicons were then digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h. ITS amplicons, as well as the corresponding restriction fragments, were analysed on agarose gel using 1.5% and 3% (w/v) agarose in 1× TBE (89 mM Tris-borate, 2 mM EDTA pH 8) buffer, stained with SYBR safe DNA gel stain (Invitrogen, Milan, Italy), visualized by UV transillumination and acquired by Gel Doc 1000 Video Gel Documentation System (BioRad, Richmond, CA). Standard DNA ladders were 1 kb Plus and 50 bp (Invitrogen). At least one isolate per group was further processed by sequencing of the D1/D2 region of the 26S rRNA gene (Gaglio et al., 2017). The identities of the generated sequences were determined by BLASTN (http://www.ncbi. nlm.nih.gov).

2.2.3. Genotypic and technological characterization of *H. uvarum* strains

2.2.3.1. Strain typing of H. uvarum isolates

The intraspecific characterization of the isolates was carried out by different RAPD-PCR assays with primers M13 (Francesca et al., 2014) and P80 (Capece et al., 2005). The same isolates were subjected to tandem repeat-tRNA (TRtRNA)-PCR method for the molecular typing of non-*Saccharomyces* subspecies, combining TtRNASc primer with either ISS-MB or $(CAG)_5$ primers, as reported by Barquet et al. (2012). All patterns were analysed using the Gelcompare II software version 6.5 (Applied-Maths, Sin Marten Latem, Belgium).

2.2.3.2. Hydrogen sulphide production

To evaluate the production of hydrogen sulphide (H_2S), the strains of *H. uvarum* were cultured onto bismuth sulphite agar (Biggy Agar), Wilson-Blair medium (Merck, Darmstadt, Germany; Jiranek et

al., 1995). H_2S production was estimated by colony blackening after 3 d of incubation at 28 °C, using a five-level scale: 0 = white, 1 = beige, 2 = light brown, 3 = brown, 4 = dark brown, 5 = black. *S. cerevisiae* US-05 (Fermentis, Lesaffre, France) and *S. cerevisiae* GR1 from the collection of SAAF Department, were used as negative (0 = white) and positive controls (3 = brown), as described by Araújo et al. (2018) and Francesca et al. (2010).

2.2.3.3. Ethanol tolerance

For ethanol tolerance assay, dilutions of exponential pure cultures were spotted onto Petri dishes containing MEA solid medium added with ethanol at 5 and 10% (v/v).

2.2.3.4. Cross resistance to hop and ethanol

The tolerance of the *H. uvarum* strains to hop was evaluated applying the procedure illustrated by Michel et al. (2016) with the following modifications: the pure strain cultures were inoculated into a set of four 10 mL tubes containing 5 ml of sterile wort (45 min at 100 °C) prepared by mixing 10% of malt extract (Oxoid, Milan, Italy) to distilled water with a final pH 5.5; the wort was adjusted to iso-α-acid concentrations of 0, 25, 50 and 90 ppm (same value in IBU) and incubated for 72 h at of 27 °C. Additional set of four tubes containing sterile wort were adjusted to 0, 25, 50 and 90 ppm iso-α-acid and added with 5% (v/v) filter-sterilized ethanol. The growth was assessed by visual inspection (Kurtzman et al., 2011; Michel et al., 2016).

2.2.3.5. Growth kinetics in presence of hop and ethanol

The growth of the pure strain cultures in wort was further investigated by optical density (OD) measurement at 600 nm wavelength into a 96-well microtitre plate (Michel et al., 2016). The strains for growth kinetics experiments were cultivated as reported by Hall et al. (2014). The measurement was performed at 1 h interval for the 72 h by using the ScanReady Microplate photometer P-800 (Life Real Biotechnology Co., Ltd, Hangzhou, China). The temperature was set at 27 °C (Salvadò et al., 2011). All analyses were performed in triplicates in two independent experiments.

The variables describing the growth curves were: total growth calculated as the integrated area underlying the curve up to 72 h; lag phase time was statistically estimated as the duration of the growth lag phase (Hall et al., 2014); the slope of exponential phase was calculated by ratioing the increase in OD versus time and expressing the angular coefficient as a percentage, as described by Hall et al. (2014); exponential phase refers to the integrated area underlying the curve and the time of exponential phase was statistically determined; the maximum growth was represented by the highest values of OD measured up to 72 h of incubation.

2.2.3.6. Flocculation assay

Flocculation assay was carried out as previously described by Tofalo et al. (2014). The medium used was Yeast Nitrogen Base (YNB, Difco Laboratories, Detroit, Mich., USA) with 2% glucose added and prepared according to manufacturer's instructions. A volume of 5 mL of this medium was inoculated with a loopful of the pure yeast in exponential growth phase and incubated at 28 °C under stationary conditions. Flocculation ability, evaluated by visual inspection and compared with appropriate controls, was graded on a scale from 0 (non-flocculent) to 5 (highly flocculent). The visual inspection was performed after 2, 15 and 20 d from strain culture inoculation. Flocculation measurement with Helm's assay was also performed. Briefly, the sedimentation of yeasts was observed in a calcium sulphate solution buffered at pH 4.5, quantifying the sedimentation volume and flocculation type according to Casey et al. (1994). All analyses were performed in triplicates.

2.2.3.7. Wort fermentation: monitoring of weight loss and strain concentration

A laboratory-scale fermentation was performed to evaluate the ability of the strains to ferment a beer wort. To guarantee standardized conditions for all trials, wort fermentation medium was prepared as described by Holt et al. (2018), with some variations: 150 g of dry Malt Extract (Munton spraymalt 6.5 EBC, Suffolk, England) and 50 ppm of calcium carbonate were added to 1 L of distilled water. The pH value was set at 5.2 with 1 mol/L HCl. Hopping was performed after autoclaving by adding an aliquot of iso-α-acid to reach a final concentration of 25 ppm (same value in IBU) (Michel et al., 2016). The final gravity of the malt extract medium was 13.3 °P. Aliquots of 150 mL of wort were put into 300 mL flasks, sealed with a Müller valve to allow the CO_2 produced during fermentation to leave the system and autoclaved at 110 °C for 15 min. After autoclaving, the malt extract wort was let to cool down to 18 °C and, subsequently, inoculated with each yeast strains. The fermentation was performed at 18 °C under static conditions and was monitored daily by measuring the weight loss up to day 12. Beer wort inoculated with *S. cerevisiae* strain US-05 was the positive control trial. The un-inoculated beer wort represented the negative control trial. According to Ciani and Maccarelli (1998), fermentation rate (FR) was calculated as daily CO_2 production after 3 d and at the end of AF. All beer wort fermentations were performed in triplicates in two independent experiments.

All experimental trials were subjected to sampling before and after the inoculation of the strains, as well as at day 3, 6, 9, and 12 of fermentation. The samples were immediately subjected to microbial counts, performed on WL nutrient agar as previously described. After growth, all isolates were picked up from the agar plates, purified to homogeneity and subjected to genotypic identification at species and strain level as reported above. All analyses were performed in duplicates.

2.2.4. Beer production

2.2.4.1. Experimental design and sample collection

To better understand the effect of *H. uvarum* inoculum during fermentation, experimental top-fermented beers were produced at a medium-scale level (10 L batch) using four different inoculum combinations for *H. uvarum* YGA34 and *S. cerevisiae* commercial strain US-05. Four experimental trials (T1, T2, T3 and T4) and one control trial (Tc) were inoculated as follows: co-inoculation of *H. uvarum* strain YGA34 and S. *cerevisiae* strain US-05 with 1:1 ratio (trial T1); co-inoculation of *H. uvarum* strain YGA34 and S. *cerevisiae* strain US-05 with 10:1 ratio (trial T2); sequential fermentation starting from YGA34 strain and, after 48 h, inoculation of US-05 strain (trial T3); monoculture of *H. uvarum* strain YGA34 strain (trial T4); monoculture of S. *cerevisiae* US-05 strain (trial Tc). The inoculation rate was planned with a cell density of 2.0×10^6 cells/mL of each yeast strain for the trials T1, T3 and T4, while *H. uvarum* YGA34 and *S. cerevisiae* US-05 were inoculated at 2.0×10^6 and 2.0×10^5 cells/mL, respectively, in trial T2.

The beers were produced at the pilot plant of SAAF Department. Brewing was performed using an "all-in-one" microbrewery plant Klarstein mod. 10031629 (Chal-Tec GmbH Berlin, Germany). Nine kilograms of Pilsen malt (BestMalz, Heidelberg, Germany), previously ground through a two roller mill (Brouwland, Beverlo, Belgium), were added to 30 L of water with 10 g of calcium chloride ($CaCl_2$) for pH correction (Marconi et al., 2016). The mash was heated to 70 °C for 40 min for the single-step mash, until the complete conversion of sugars, verified with iodine solution. Subsequently, the mixture was heated to 78 °C for 10 min. The grains were rinsed (sparging) using 20 l H_2O, resulting in a total volume of 45 l. The resulting wort was boiled for 60 min, during which the hops (pellets, 40 g, 11.5% of α-acids) were added to reach a final concentration of 25 IBU. After boiling, the resulting volume was 42 l, with 13.5 °Bx (Brix degree). The wort was then clarified through a whirlpool consisting of 10 min of recirculation and 10 min of resting (Marconi et al., 2016). The must was finally cooled to 21 °C in preparation for the inoculation of the selected yeast strains. Standard quality parameters of beer wort were: 5.62 pH, 13.5 °Bx, 1053 SG (Specific Gravity), 7.16 g/L D-glucose, 0.94 g/L D-fructose, 10.02 g/L sucrose, 75.63 g/L maltose, 71.4 mg/L free α-amino nitrogen, 106.6 mg/L ammonia nitrogen.

Strains were reactivated from - 80 °C glycerol stocks and plated onto YPD agar (10 g/L yeast extract, 20 g/L bacteriological peptone, 15 g/L agar, and 20 g/L D-glucose). After 3 – 5 d at 25 °C, the strains were pre-cultured overnight in 5 mL YPD and then re-inoculated into sterile flasks containing YPD, where cells were allowed to grow for 3 d at 25 °C (Holt et al., 2018). The cells were washed twice with Ringer solution and inoculated as described above.

At the end of AF, the beer samples were bottle conditioned for 16 d by dextrose addition (4.5 g/L) and US-05 with a pitching rate of 5.0×10^6 cells/mL.

All experimental fermentation trials were performed in duplicate. Samples were collected at different stages of beer production: wort, after the inoculum of yeast strains, during the AF (day 1, 2, 4, 6 and 11), at the end of AF (green beer), at the end of bottle conditioning. All samples were subjected to analysis within 24 h from collection. All analyses were performed in triplicate.

2.2.4.2. Microbiological counts, isolation and genotype monitoring of the added strains

The concentration of yeasts (total yeast, *Saccharomyces* spp. and *Hanseniaspora* spp. populations) were evaluated onto WL nutrient agar (Martin et al., 2018). After growth, all isolates were picked up from the agar plates, purified to homogeneity and subjected to genotypic identification as reported above (paragraph 2.2.). To verify the dominance of *H. uvarum* strain YGA34, all isolates within *Hanseniaspora spp.* group were characterized at strain level as reported above (paragraph 2.3.1.).

The genetic diversity of *Saccharomyces* isolates was assessed by Interdelta analysis (Legras and Karst, 2003). Interdelta patterns were analysed using the Gel Compar II software (v. 6.1. Applied Maths NV. Sint-Martens-Latem. Belgium) and similarities among patterns were assessed. Profiles showing more than 95% of similarity were considered identical.

2.2.4.3. Determination of physicochemical parameters

The pH measurement was conducted with a pH meter (Mod.70 XS/50010162) while °Bx were determined with a refractometer (DBR Salt). The analyser iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co.. Ltd.. Shenzhen, China) was used and run with full automation for the enzymatic determination of glucose, fructose, sucrose, maltose, ethanol, glycerol, and acetic acid. It automatically pipetted reagents and samples into the cuvette, allowed incubation at a controlled temperature, read absorbance at the specific wavelength, and calculated the concentration of the analyses with a calibration curve. The parameters used in the automated photometric systems were: temperature, 37 °C; wavelengths, 340 nm and 415 nm (bichromatic); optical path. 1 cm. The reagents used were: Enzytec™ Liquid D-Glucose / D-Fructose Cod. E8160. Enzytec™ Liquid Sucrose/D-Glucose Cod. E8180. Enzytec™ Liquid Ethanol Cod. E8340. Enzytec™ Fluid Glycerol Cod. E5360. Enzytec™ Acetic acid Cod. E2580. The standard used for the calibrations of the apparatus were: Enzytec™ Multi-acid standard automation Cod. E1241 for acetic acid; Enzytec™ Sugar standard Cod. E5450 for glucose and fructose; Enzytec™ Alcohol standard Cod. E5420 for ethanol; Enzytec™ Sugar standard manual Cod. E1242 for glycerol. All reagents and standards were purchased from R-Biopharm AG (Darmstadt. Germany). All samples were diluted to the optimal concentration with respect to the calibration curve of the apparatus.

2.2.4.4. Determination of volatile organic compounds

The volatile compounds of the experimental beers were determined in duplicate using an Agilent Technologies 6850 gas chromatograph (GC) equipped with an Agilent Technologies Mass Spectrometer (MS) 5975C (Santa Clara, CA, USA), The GC-MS with a glass direct inlet liner (1.5 mm inner diameter and 140 μL volume) and a DB-5MS capillary column of 60 m × 0.32 mm × 1 μm (J&W Scientific, Inc., Folsom, CA, USA) consisting of cross-linked 5% phenyl methyl siloxane was used. Typical beer volatile compounds were determined according to Vesely et al. (2003) and Malfliet et al. (2009) based on solid-phase microextraction (SPME) with on-fibre derivatization using a 65 μm PDMS/DVB fiber coating (Supelco, Bellefonte, PA, USA; catalogue no. 57328-U). The analyses were performed in triplicate. An aqueous solution (4 g/L) of the derivatization agent O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBOA, Sigma-Aldrich, Milwaukee, WI, US) was prepared each week. The internal standard for the determination of aldehydes and vicinal diketones, 2-chlorobenzaldehyde (Sigma-Aldrich Milwaukee, WI, US), was prepared weekly in a solution of 5% ethanol with a concentration of 10 mg/L. The internal standard for the determination of higher alcohols and esters was 1-butanol (Sigma-Aldrich), which was prepared each week in water at a concentration of 60 mg/L. The analyses were conducted in triplicate.

2.2.4.5. Sensory analysis

The designed sensory evaluation of experimental beers consisted of quantitative descriptive analysis carried out by panellists to define colour, odour, taste and overall quality.

Eleven judges (ranging from 23 to 52 years old) were recruited from University of Palermo, beer associations and professional brewers. All had experience in brewing and participated in previous studies as sensory judges. The judges were submitted to preliminary tests to determine their sensory performance on basic tastes and the aromas associated with beers. The sensory analysis of beers was conducted following the methodology reported by Marconi et al. (2016) and ISO regulations: visual perception (appearance), olfactory sensations based on odour (via the nostril, orthonasally) and flavour (via the back of the throat, retronasally), oral sensations based on taste, mouthfeel and overall quality. The odour was explained as perception of volatile compounds with the beverage outside the mouth [orthonasal (in-glass) odour]; the flavour as perception of volatile compounds with the beverage inside the mouth and back of the throat [retronasal (mouth-derived) odour]; basic taste as gustatory sensations and mouthfeel sensations as chemical feeling factors with beverage inside and after the mouth and overall quality based on global evaluation of odour, taste, mouth-feel and flavour (Issa-Issa et al., 2020; Jackson, 2016).

The panellists consensually generated 28 sensory descriptive attributes regarding appearance, odour, flavour, taste and overall quality in several sessions. The set of attributes were: appearance (yellow

colour); odour (intensity, complexity, fruity, citrus, floral, hoppy, cereal/grainy, malty, honey/caramel, roasted/burnt, sulphury, acetic, oxidized/aged, alcohol, and off-odour); gustatory taste (sweet, bitter, sour and salty); mouthfeel (body and astringent); flavour (intensity, complexity, fruity, citrus, hoppy, cereal/grainy, malty, honey/caramel, roasted/burnt, alcohol, sulphury, and off-flavour. The descriptor overall quality was also included for both odour and flavour.
The panellists also generated a consensual descriptive ballot for the experimental beers in which the descriptors were associated with a 9 cm unstructured scale anchored at the left and right extremes with the terms “none/weak” and “strong”, respectively (Jackson, 2016).
The sensory assessments were performed in blind tasting conditions at the tasting room of University of Palermo (Palermo, Italy). The experimental beers samples (50 ml) were served monadically at 15 °C in standard ISO type tasting glasses, labelled with three-digit random codes. Water was provided for rinsing between beers. All evaluations were made between 10.00 and 12.00 a.m. in individual booths (ISO 8589, 234 2007). The final scores were obtained as a mean of three evaluations with the respective statistical analysis.

2.2.5. Statistical and explorative multivariate analyses

Data were investigated using a generalized linear model (GLM) based on ANOVA model that included effects of *in vitro* tests and strain, as well as the interaction between tests and strains. The post-hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to $p < 0.05$ (Mazzei et al., 2010). In addition, explorative multivariate analysis was conducted to better differentiate the strain combinations on the basis of the results from *in vitro* technological screening, micro-fermentations and laboratory-scale beer productions.
In details, the statistical analyses were performed on the data set including a total of 58 variables: total growth, exponential growth, maximum growth, lag phase time, slope of exponential phase calculated in absence of hop and ethanol, as well as in presence of different concentrations of hop (25, 50 and 90 IBU) and ethanol (5% v/v); resistance to ethanol (5, 10% v/v); resistance to hop (25, 50 and 90 IBU); cross resistance both to ethanol (5% v/v) and hop (25, 50 and 90 IBU); production of H_2S and flocculation growth pattern. The agglomerative hierarchical cluster analysis (AHC) and principal component analysis (PCA), based on correlation matrix by Pearson (n), and implemented by symmetric biplot representation were performed in order to investigate relationships among the samples as reported by Martorana et al. (2015). Due to high number of variables describing the technological screening of strains, a graphical representation of individual values and distribution of technological characteristics among strains was performed by using a heat map clustered (HMC). This analysis was based on double hierarchical dendrogram with heat map plot, the individual content values contained in the data matrix as colours. The relative values of the technological characteristics

were depicted by colour intensity from yellow (lowest value) to red (highest value). HMC analysis of values was performed using the autoscaled data. Data set resulted from VOCs and sensory analysis of experimental beers produced at laboratory-scale was also subjected to explorative multivariate analysis (HCA, PCA and HMC) following the same methodology reported above.

All data subjected to multivariate analysis were preliminary evaluated by using the Barlett's sphericity test (Dillon and Goldstein, 1984; Mazzei et al., 2013) to check the statistically significant difference among strains within each data set. STATISTICA software v.10 (StatSoft Inc., Tulsa, OK, USA) was used for data processing and graphic construction of HCA and PCA analyses. The XLStat software version 7.5.2 (Addinsoft, New York, USA) was applied for HMCA and biplot representation.

2.3. RESULTS

2.3.1. Microbiological analysis of FHP samples

Yeast loads in the 12 FHP samples analysed are reported in Table 1. Significant differences in terms of yeast cell densities were registered among samples and media used for colony enumeration. The highest yeast counts were found on WL agar (5.9 Log CFU/ml). The levels of non-*Saccharomyces* population detected on LA was quite variable ranging between 2.23 and 6.01 Log CFU/ml. Sample FHP-3 showed the highest non-*Saccharomyces* levels on both media. In general, the lowest levels were registered for total osmophilic and osmotolerant yeasts on DWA and TGYA media, respectively.

Table 1. Microbial loads (Log CFU/g or ml) of fermented honey by-products (FHP) samples

FHP Samples	Media			
	DWA	TGYA	WL	LA
1-a	$2.98 \pm 0.27^{a,b}$	$3.01 \pm 0.32^{b,c,d}$	$5.45 \pm 0.22^{a,b}$	4.88 ± 0.22^{d}
1-b	$2.73 \pm 0.17^{a,b,c}$	$3.47 \pm 0.11^{a,b}$	$4.75 \pm 0.41^{b,c,d,e}$	$5.02 \pm 0.33^{c,d}$
1-c	2.29 ± 0.28^{c}	$2.8 \pm 0.17^{c,d,e}$	$4.88 \pm 0.49^{b.c.d.e}$	$5.17 \pm 0.21^{b,c,d}$
2-a	$2.44 \pm 0.31^{b,c}$	2.22 ± 0.19^{e}	$5.33 \pm 0.31^{a,b,c,}$	2.88 ± 0.28^{e}
2-b	$3.04 \pm 0.21^{a,b}$	$2.74 \pm 0.34^{c,d,e}$	5.87 ± 0.31^{a}	3.01 ± 0.21^{e}
2-c	$2.72 \pm 0.20^{a,b,c}$	$2.69 \pm 0.24^{d,e}$	5.92 ± 0.37^{a}	2.23 ± 0.21^{e}
3-a	n.d.^{d}	n.d.^{f}	$5.45 \pm 0.40^{a,b}$	6.01 ± 0.30^{a}
3-b	n.d.^{d}	n.d.^{f}	$5.17 \pm 0.32^{a,b,c,d}$	$5.72 \pm 0.48^{a,b,c}$
3-c	n.d.^{d}	n.d.^{f}	$5.08 \pm 0.17^{a,b,c,d,e}$	$5.89 \pm 0.20^{a,b}$
4-a	3.18 ± 0.17^{a}	$3.33 \pm 0.38^{a.b.c}$	4.15 ± 0.20^{e}	4.88 ± 0.22^{d}
4-b	$2.95 \pm 0.41^{a,b}$	3.87 ± 0.15^{a}	$4.25 \pm 0.31^{d,e}$	$5.02 \pm 0.33^{c,d}$
4-c	3.14 ± 0.20^{a}	3.8 ± 0.11^{a}	$4.38 \pm 0.31^{c,d,e}$	$5.17 \pm 0.21^{b,c,d}$
Statistical significance	***	***	**	***

Abbreviations: DWA, De Whalley Agar for total osmophilic yeasts; TGYA, tryptone glucose yeast extract agar for total osmotolerant yeasts; WL, Wallerstein Laboratory nutrient agar for total yeasts; LA, lysine agar for *Saccharomyces* yeast growth inhibition; n.d., not detected (value < detection limit of method). Results indicate average values standard deviation of three plate counts.

Results indicate mean values ± SD of three determinations.

Data within a column followed by the same letter are not significantly different according to Tukey's test.

P value: *, P < 0.05; **, P < 0,01; ***, P < 0.001; n.s., not significant.

2.3.2. Genotypic identification of yeasts

A total of 404 yeasts were isolated from FHP samples and subjected to genotypic characterization. The restriction analysis of ITS1-5.8S-ITS2 separated the isolates into five groups (Table 2); five groups were preliminary identified at species level by comparison of the restriction profiles with those reported in literature (Esteve-Zarzoso, et al., 1999; Francesca et al., 2014; Sannino et al., 2013). Specifically, the isolates were identified as *H. uvarum* (group I), *S. cerevisiae* (group II), *Wickerhamomyces anomalus* (group III), *Zygosaccharomyces bailii* (group IV) and *Zygosaccharomyces rouxii* (group V). The genotypic identification of yeasts was completed by pairwise alignment of D1/D2 sequence of the type strain of each species (Table 2).

Table 2. Molecular identification of yeast species isolated from FHP samples

Species	Profile	5.8S-ITS PCR	Size of restriction fragment[1]				Accession number[2]	No. of isolates[3] (%)[4]
			*Cfo*I	*Hae*III	*Hinf*I	*Dde*I	bp	
Hanseniaspora uvarum	I	750	320+105	750	350+200+180	300+180 +95+90	MT362721; MT362722	145 (36)
Saccharomyces cerevisiae	II	850	370+340	320+220 +180+130	380+120	n.d.	MT364261; MT364262	51 (13)
Wickerhamomyces anomalus	III	650	575	600+50	310+310	n.d.	MT364263; MT364264	43 (11)
Zygosaccharomyces bailii	IV	775	330+295	700	340+230+175	n.d.	MT364265	88 (22)
Zygosaccharomyces rouxii	V	735	295+205 +175	420+175	350+250+130	n.d.	MT364266	77 (19)

[1] Values refer to the number of base pairs (bp) per fragment.
[2] Accession number of D1/D2 region of the 26S rRNA gene of isolates deposited into Genbank database
[3] Number of isolates per each yeast species.
[4] Percentage based on the total number of isolates.
Abbreviation: n.d., not determined according to Esteve-Zarzoso et al. (1999).

With regards to genera/species distribution (Table 2) among samples, the majority of isolates belonged to the *H. uvarum* group. For this reason, isolates belonging to group I were subsequently screened for brewing parameters. The species *Z. bailii* and *Z. rouxii* were also isolated from several samples and their presence reached 22 and 19% of total number of isolates, respectively.

All isolates of *H. uvarum* were further investigated at strain level by RAPD-PCR and TRtRNA-PCR analysis. The dendrogram resulting from these analyses (Fig. 1) showed that these isolates represented five distinct clusters corresponding to five different strains (YGA2, YGA34, YGA36, YGA38 and YGA73).

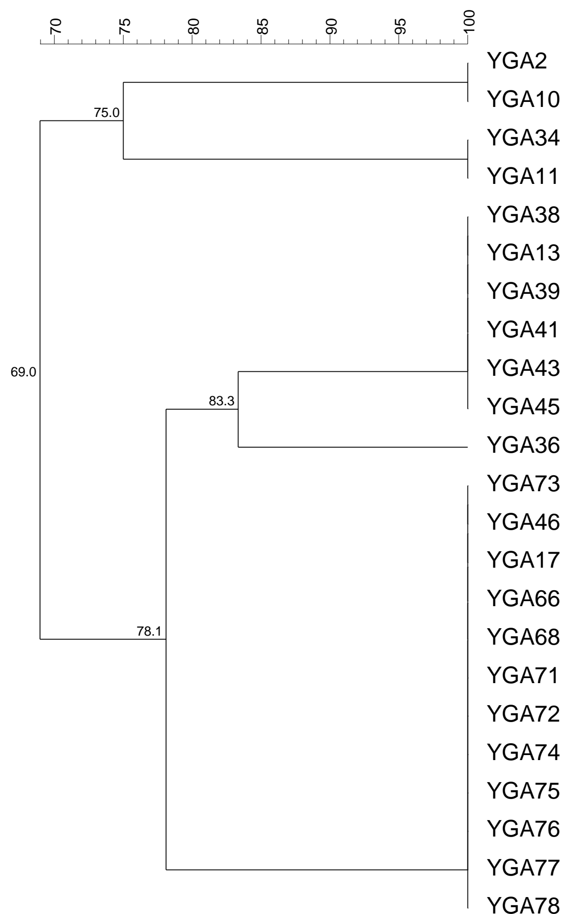


Figure 1. Dendrogram obtained by combination of RAPD profiles with M13 and P80 primers and TRtRNA typing generated with the primers TtRNASc, ISS-MB and $(CAG)_5$ from *H. uvarum* isolates. The scale indicates the Dice similarity coefficient.

2.3.3. Technological characteristics of *H. uvarum strains* for beer production

2.3.3.1. H_2S production and flocculation tests

The five *H. uvarum* strains were screened for their brewing characters (Table 3). All strains were characterized by a very low production of H_2S on Biggy agar plates (white - light brown colony) and non-flocculent behaviour. The mean sedimentation volume measured with Helm's assay ranged between 0.35 and 0.7 mL. According to Casey et al. (1994), all yeasts showed a type II flocculation with a rising interface near the bottom of the testing tubes, typical of non-flocculent yeasts.

2.3.3.2. Resistance to ethanol and hop

Intense growth at 5% (v/v) of ethanol was observed for all strains, while only a weak growth was found for the strains YGA2, YGA34, YGA36 and YGA38 at 10% (v/v). Only the strain YGA73 was unable to grow at 10% (v/v) of ethanol (Table 3).

The growth in presence of iso-α-acid was also evaluated (Table 3). All strains were able to grow in liquid medium containing 0, 25, 50 and 90 IBU. In terms of cross-resistance to ethanol and hop, all

H. uvarum strains were able to growth in presence of 5% ethanol and up to 25 IBU (Table 3). The strains YGA36 and YGA38 showed growth at 5% (v/v) ethanol and up to 90 IBU.

Table 3. Technological screening of *H. uvarum* strains

Strain	Resistance to ethanol		Resistance to hop			Cross resistance			H_2S[a]	Flo[b]	Sed[c]
	5% (v/v)	10% (v/v)	25 IBU	50 IBU	90 IBU	25 IBU/ 5% ethanol	50 IBU/ 5% ethanol	90 IBU/ 5% ethanol			
YGA2	+	+/-	+	+	+	+/-	-	-	0	0	0.55
YGA34	+	+/-	+	+	+	+	+/-	-	0	0	0.65
YGA36	+	+/-	+	+	+	+	+/-	+/-	1	0	0.60
YGA38	+	+/-	+	+	+	+	+/-	+/-	0	0	0.35
YGA73	+	-	+	+	+	+	-	-	0	0	0.45

Symbols: +, positive growth; −, no growth; +/-, weak growth;
Abbreviations: IBU, International Bitterness Unit; H_2S, Hydrogen sulphide;
[a] Colour of colony on Biggy agar plates: 0 = white; 1 = beige; 2 = light brown; 3 = brown; 4 = dark brown; 5 = black.
[b] Flocculation degree after 22 days of incubation.
[c] Mean sedimentation volume (mL) expressed according to Helm's Assay

2.3.3.3. Growth kinetics in presence of hop and ethanol

The resistance to ethanol and hop was further investigated by dynamic measurement of cell growth kinetics at 600 nm within 72 h from inoculation (Fig. 2). The strain growth curves were analysed in terms of time of lag phase (LP), slope of exponential phase (EP). The highest scores reached during the stationary phase (SP) were also included in the analysis.

When the tests were performed at different concentrations of iso-α-acids without ethanol (Fig. 2A-D), all *H. uvarum* strains showed LP times comparable to control *S. cerevisiae* US-05, with values between 4 to 9 h. The values of EP slope in *H. uvarum* strains resulted significantly higher than control trial; the EP for the strain US-05 was, on average, 2.4%, while the *H. uvarum* strains reached values between 6.77% (YGA38) and 10.04% (YGA2). Between the 12th and 16th hour of incubation, all *H. uvarum* strains reached SP (0.72), while the strain US-05 reached 0.49 OD. From the 24th hour onwards, the control US-05 showed higher values of SP than *H. uvarum* strains, with a maximum value of 1.50 registered at the end of the monitoring. In addition, significant differences were found among the five *H. uvarum* strains when the growth curves were calculated in presence of different IBU without ethanol. The strains YG34, YGA2 and YGA73 showed the best growth within the 16th hour of incubation, with a SP value 0.2 units higher than that recorded for the strains YGA36 and YGA38. At 90 IBU, the strain YGA34 reached the highest value of EP slope (6.23%). When the strains grew in presence of both 5% (v/v) ethanol and different IBU, their LP time significantly increased, from an average of 5.9 h (without ethanol) to an average of 32 h (with 5% ethanol) (Fig. 2E-H). Once again, the best values of both LP time and EP slope were found for the strains YGA2 and YGA34.

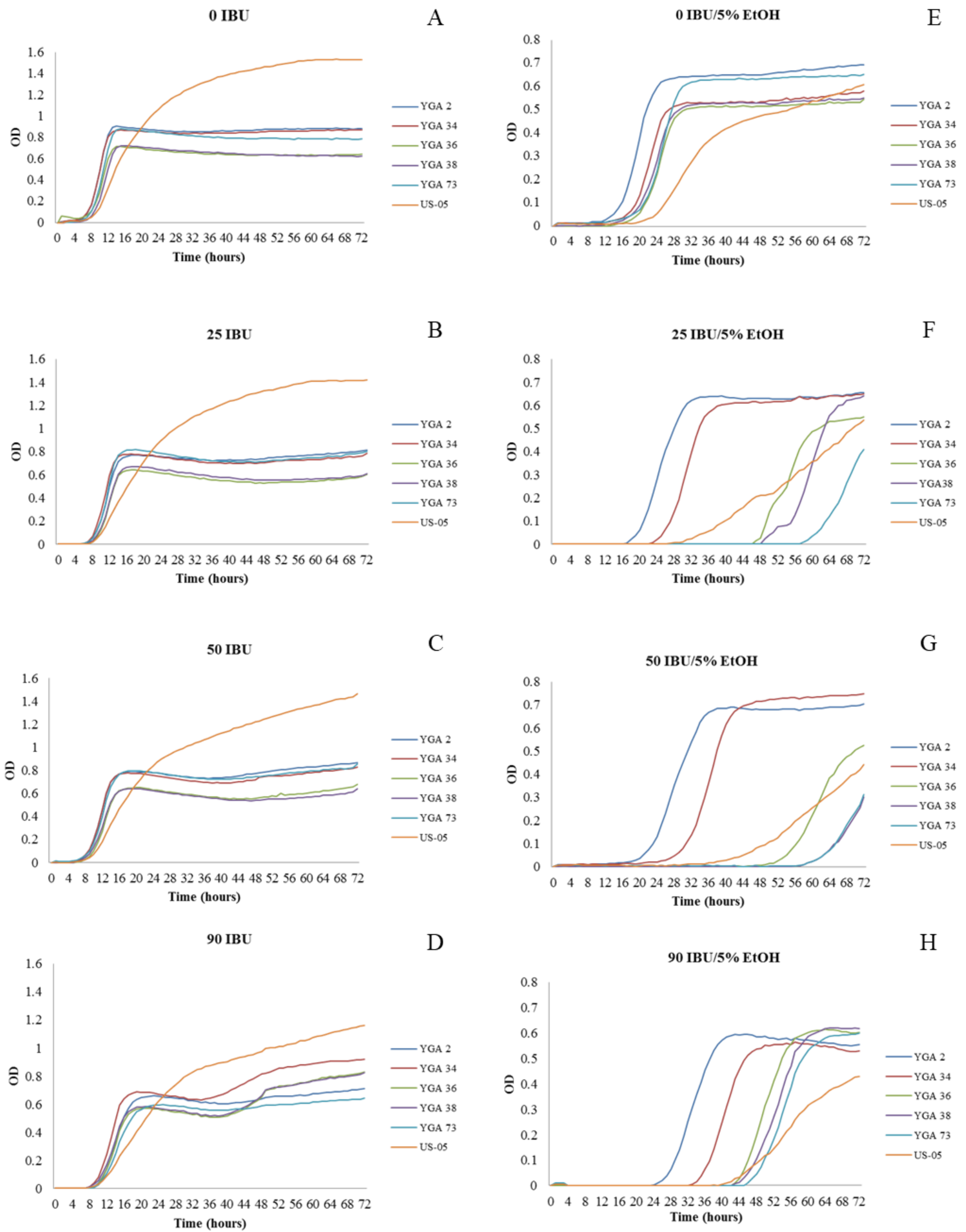


Figure 2. Growth of *H. uvarum* strains at different IBU and ethanol concentrations. Abbreviation: IBU, International Bitterness Unit; OD, optical density. The sensitivity to different IBU concentration were measured by OD values at 600 nm in triplicate. Values of standard deviations ranged between 0 and 0.315 and are not showed for a better graphical visualization of figures.

In detail, LP time for these strains ranged between 10 and 33 h, while that of the control strain ranged between 19 and 39 h, depending on IBU concentration. When, the IBU concentration increased up to 50 and 90 the differences between the strains YGA2 and YGA34 and the control strain increased significantly (Fig. 1G-H), with a LP time 13 h shorter and SP values higher (0.74 for YGA34 and 0.68 for YGA2) than those observed for the strain US-05, which obtained values of 0.44. Statistical significance was attributed to $p < 0.05$ (Mazzei et al., 2010).

2.3.3.4. Wort fermentation

The kinetic of weight loss due to CO_2 production is graphically presented in Figure 3.

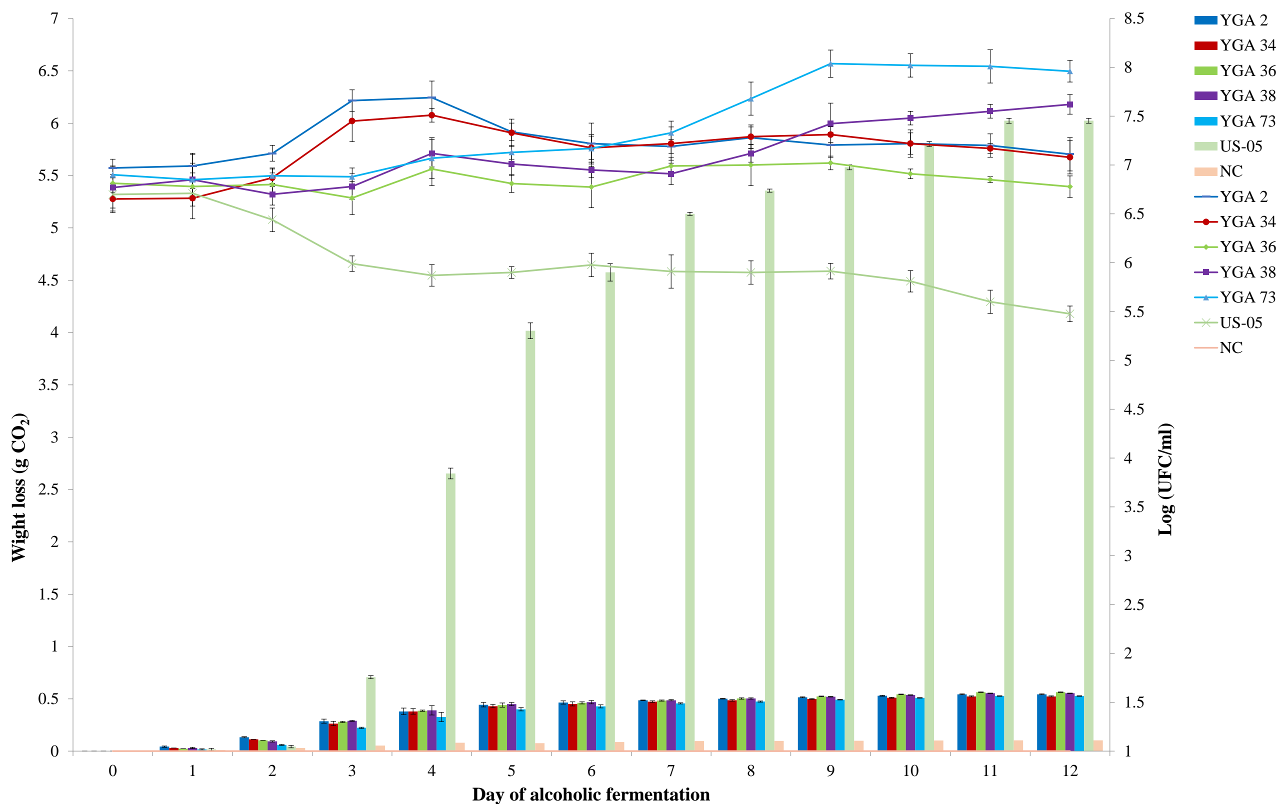


Figure 3. Fermentation kinetics of wort beer inoculated with *H. uvarum* strains. Axes: values of principal vertical axis (left side) refer to histogram graph and represent the weight loss (g CO_2) of wort beer during AF; values of secondary vertical axis (right side) refer to growth curve of strains inoculated into wort beer during AF.
Codes: YGA2, YGA34, YGA36, YGA38 and YGA73, are codes of *H. uvarum* inoculated into wort representing the experimental trials; US-05, code of *S. cerevisiae* commercial strain inoculated into wort representing the positive control trial; NC, negative control trial subjected to spontaneous fermentation.

The values of FR at 3 d for *H. uvarum* strains ranged between 0.22 and 0.29 g. The positive control trial, inoculated with *S. cerevisiae* strain US-05, showed the highest FR (0.71 g). At day 12 of AF, all *H. uvarum* strains showed a FR between 0.52 and 0.56 g. The highest value of FR was found for the control strain US-05 (6.02 g).

During fermentation, the strains were also monitored by microbiological analysis (Fig. 2). Just after inoculation, all strains were found at concentrations ranging between 6 and 7 Log CFU/ml. At day 3

of AF, all *H. uvarum* strains reached levels higher than those of the control strain US-05; the highest counts (7.40 Log CFU/ml) were displayed by the strains YGA2 and YGA34. From day 6 onward, the levels of all *H. uvarum* strains were about 6.5 Log CFU/ml which was higher than the level registered for the control strain US-05. At day 12, the end of monitoring, all experimental trials showed an increase of yeast levels; the highest values, 7.42 and 8.03 Log CFU/ml, were found for the strains YGA38 and YGA73, respectively.
The persistence of the strains inoculated was phenotypically and genotypically investigated. All isolates collected from the experimental trials showed shape of colony and cellular morphology typical of *Hanseniaspora* genus (Cadez et al., 2014; Jindamorakot et al., 2009; Kurtzman et al., 2011). In addition, the dendrogram resulting from RAPD-PCR analysis showed that all isolates were divided into five clusters for the five strains inoculated. All isolates collected from control trial shared the same RAPD pattern of *S. cerevisiae* strain US-05 (data not shown).

2.3.5. Beer production

2.3.5.1. Yeast growth during fermentation

The theoretical inoculum ratio resulted as planned, with one Log cycle difference between the two yeast species for the trial T2 and an inoculum of about 6 Log cycle for the other trials. The ratio between the two yeast species involved in trials fermentation is reported in Figure 4.
Trial T1, inoculated with *S. cerevisiae/H. uvarum* ratio of 1:1, showed similar levels of the two species (6.5 Log CFU/mL for US-05 and 6.3 Log CFU/mL for YGA34) at t_0. This ratio was maintained also at 24 h after inoculum, but a 0.5 Log cycles increase of *S. cerevisiae* over *H. uvarum* was registered at 48 h. After 4 d of AF, a decrease of cell density was registered for *S. cerevisiae*, while the count resulting for *H. uvarum* were quite constant.
Trial T2 revealed a complete dominance of the strain YGA34 during fermentation. For this trial, the levels of *H. uvarum* were 0.8 - 1.6 Log cycles higher than those of *S. cerevisiae* for the entire fermentation. The highest levels of *H. uvarum* YGA34 was reached at 2 and 6 d of AF, with 7.8 Log CFU/mL, while the strain US-05 reached 6.8 Log CFU/mL at the 6th day.
Regarding trial T3, the strain YGA34 showed an increase of cell density during the first 4 d of AF, with values from 6.3 to 7.7 Log CFU/mL; after day 4, a slight decrease to 7.5 Log CFU/mL at day 11 was registered. After the inoculum of US-05 at 48 h, *S. cerevisiae* population showed the maximum cell density at day 6 of AF (6.7 Log CFU/mL) while the lower value was registered at day 11 of AF (5.9 Log CFU/mL).

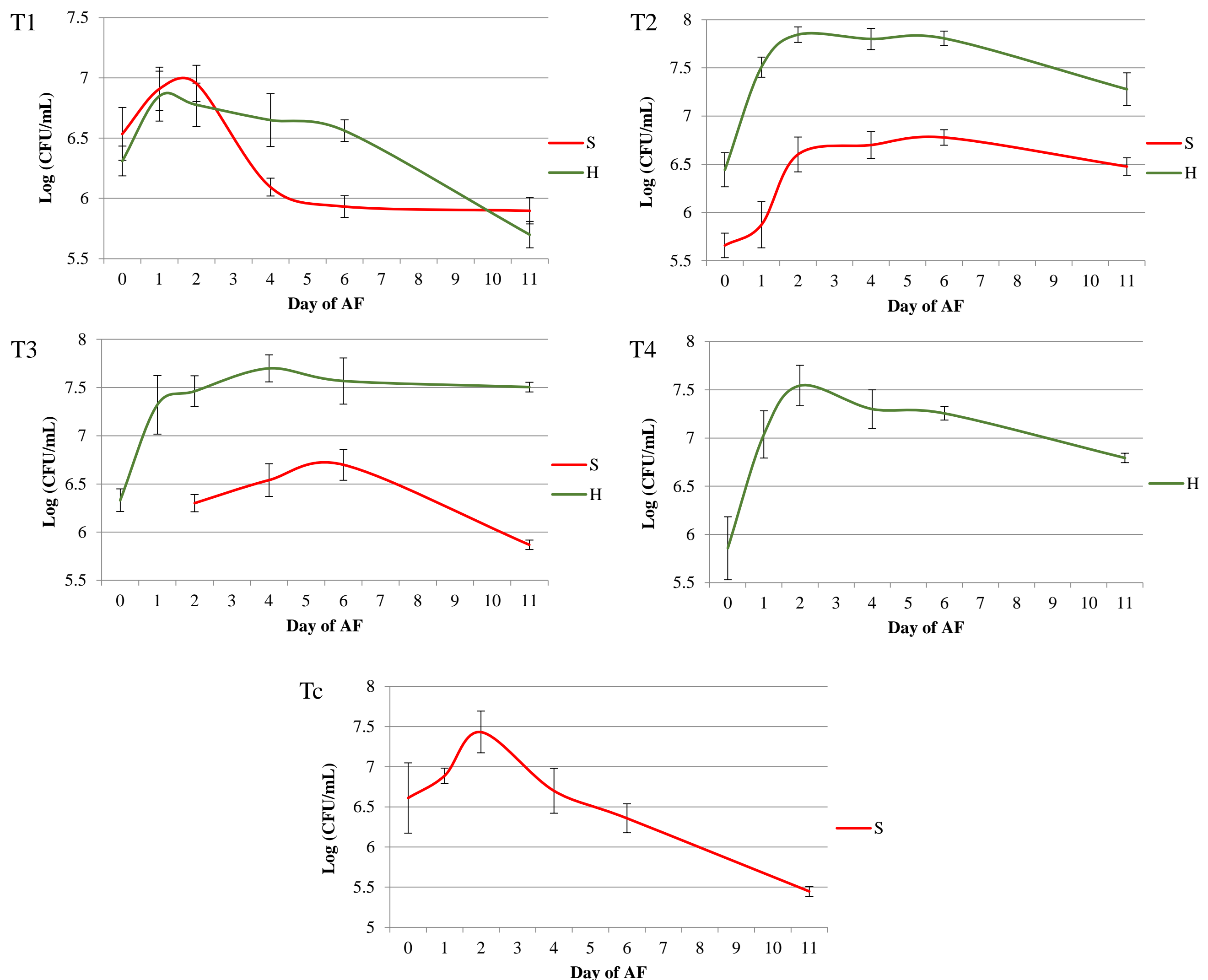


Figure 4. Evolution of yeast populations of *S. cerevisiae* and *H. uvarum* populations during trials fermentation.
Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; T4 refers to fermentation trial inoculated with monoculture of YGA34; Tc refers to fermentation trial inoculated with monoculture of US-05. Legend: -S refers to *S. cerevisiae* population; -H refers to *H. uvarum* population.

Trial T4, inoculated only with *H. uvarum*, although characterized by an initial load of 5.5 Log CFU/mL, grew rapidly up to 7 Log cycles 24 h after the inoculum and maintained these values until day 6 of AF; at day 11 of monitoring, the yeast population of *H. uvarum* was 6.8 Log CFU/mL.
The control trial displayed a classic fermentation kinetics.

2.3.5.2 pH, total soluble solids and sugar changes during fermentation

At the end of AF, pH values ranged between 3.95 and 4.08 for all trials except for T4 and un-inoculated control, which showed higher values of 4.87 and 5.48 respectively.
The final gravity (FG) of the beers indicated similar value for thesis T1, T2, T3 and Tc, with 1.015, while a value of 1.050 was registered for T4, with a decrease of 0.002 SG points respect to initial wort. The SG of the non-inoculated must did not show any detectable decrease during fermentation.

The percentages of the residual sugars are reported in Table 4.

Table 4. Mean percentage of sugar consumption during AF calculated for each trial.

	D-glucose (%)	D-fructose (%)	Sucrose (%)	Maltose (%)
T1	99.4 ± 0.02	97.3 ± 0.05	100 ± 0.05	82.0 ± 0.04
T2	99.5 ± 0.05	97.9 ± 0.01	100 ± 0.07	89.2 ± 0.10
T3	99.5 ± 0.02	98.1 ± 0.07	100 ± 0.49	84.6 ± 0.03
T4	99.2 ± 0.03	96.8 ± 0.02	37.9 ± 0.39	2.2 ± 0.07
Tc	99.4 ± 0.05	96.8 ± 0.08	100 ± 0.07	84.3 ± 0.01

Results indicate mean values ± SD of three determinations.

Fructose and glucose were fermented to over 96% in all experimental trials. Except in T4, sucrose was completely fermented for all other trials. Maltose consumption ranged between 82.0 and 89.2 % for the trials T1, T2, T3 and Tc. The trial T4, inoculated only with monoculture of *H. uvarum*, did not show any maltose fermentation due to the inability of this species to use maltose; for this reason, bottle conditioning was not carried out for trial T4.

In terms of ethanol, glycerol and acetic acid production measured at the end of AF, the values are reported in Table 5.

Table 5. Final concentration of ethanol, glycerol and acetic acid in green beers produced with different combinations of inoculum

	Ethanol (%)	Ethanol yield (g/g)	Glycerol (g/L)	Acetic acid (g/L)
T1	5.05 ± 0.16^{ab}	0.50 ± 0.06^{a}	3.20 ± 0.18^{b}	0.03 ± 0.02^{c}
T2	5.16 ± 0.13^{a}	0.48 ± 0.04^{a}	3.09 ± 0.08^{b}	0.17 ± 0.03^{b}
T3	4.80 ± 0.07^{c}	0.46 ± 0.04^{a}	3.80 ± 0.06^{a}	0.26 ± 0.04^{a}
T4	0.52 ± 0.01^{d}	0.31 ± 0.02^{b}	1.28 ± 0.40^{c}	0.27 ± 0.01^{a}
Tc	4.90 ± 0.11^{bc}	0.47 ± 0.05^{a}	3.08 ± 0.04^{b}	0.03 ± 0.02^{c}
Statistical significance	***	*	***	***

Results indicate mean values ± SD of three determinations.
Data within a column followed by the same letter are not significantly different according to Tukey's test.
P value: *, $P < 0.05$; **, $P < 0{,}01$; ***, $P < 0.001$; n.s., not significant

The highest value of ethanol was reached in T2, with 5.16% (v/v). Trial T4, which did not complete the AF, showed a final ethanol value of 0.52%.

Regarding glycerol content, except for T4 showing 1.3 g/L, this parameter ranged between 3.1 for T2 and Tc and 3.8 g/L for in T3. The acetic acid content detected at the end of fermentation was below 0.27 g/L for all trials. The highest value for this parameter was registered in trials T3 and T4, while the lowest value (0.03 g/L) was recorded for T1 and Tc.

2.3.5.3 Determination of volatile compounds

The assessment allowed the identification of 18 compounds, as higher alcohols, aldehydes, esters and vicinal diketones. The values of total VOCs were 198.36 mg/L (T1), 169.61 mg/L (T2), 186.28 mg/L (T3) and 225.98 mg/L (Tc).

The experimental beers were characterized by different flavour profile, as represented in heat-map (Fig. 5), in which the relationships among beers are based on the amount of each VOC. Among these volatile compound class, 2-methylpropan-1-ol dominated especially in Tc and T1 trials, followed by 3-methylbutan-1-ol and propanol. The second most abundant VOC class is represented by esters.

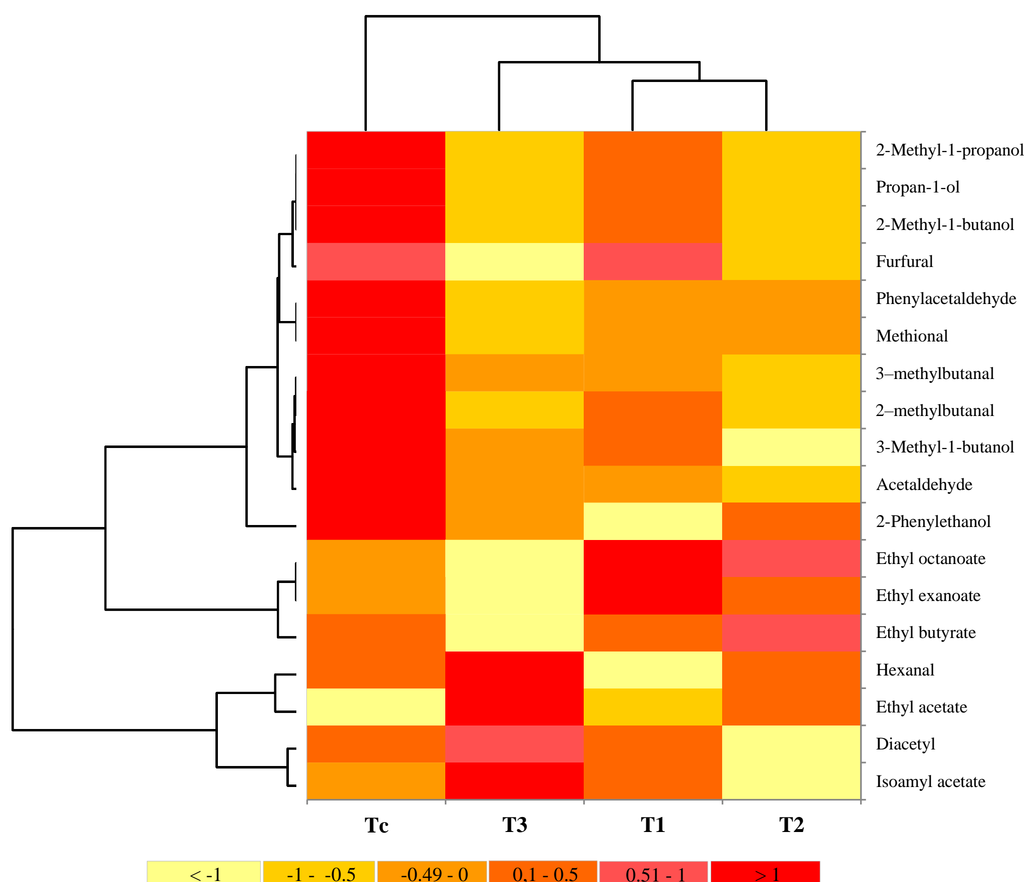


Figure 5. Distribution of volatile organic compounds among experimental beers. The heat map plot depicts the relative concentration of each VOCs (variables clustering on the Y-axis) within each sample (X-axis clustering). Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

With this regard, substantial differences were found in the overall level of these VOCs group, with maximum values recorded for trial T3 (32.7 mg/L), while the minimum value was recorded by Tc (9.2 mg/L). Except for trial T3, which reached values above this threshold (32.2 mg/L), all other trials showed lower values, with the minimum value obtained by the control trial (8.7 mg/L). In particular, isoamyl acetate ranged between 0.16 mg/L in T2 and 0.31 in T3, while ethyl hexanoate exhibited values always lower than 0.08 mg/L.

2.3.5.4. Sensory analysis

The sensory evaluation on the final products showed several statistically significant differences among trials, which are shown in Figure 6.

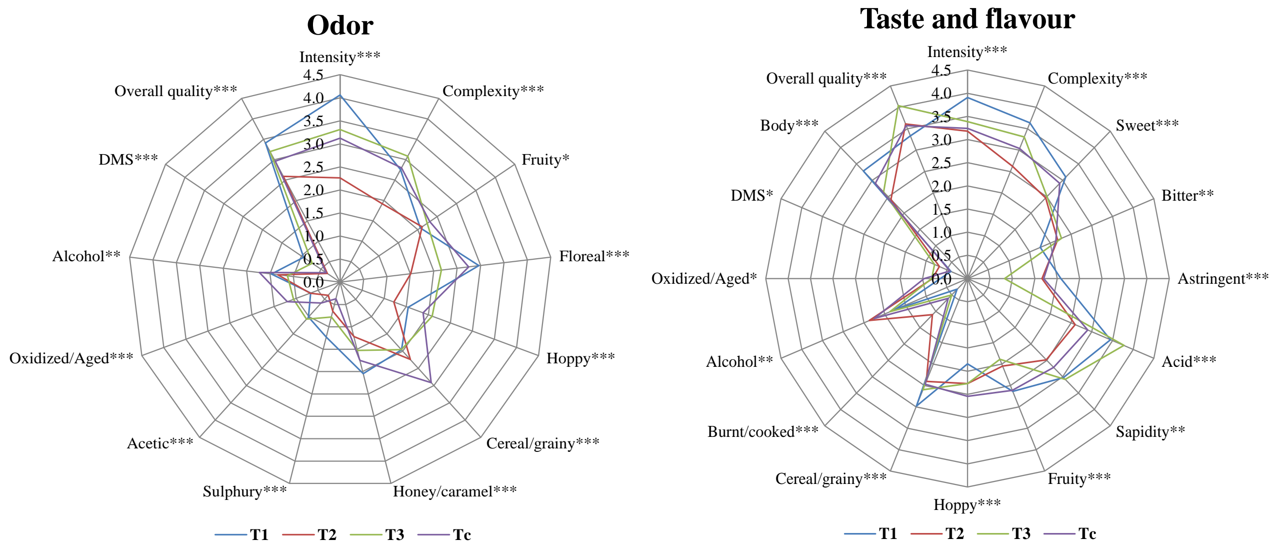


Figure 6. Spider-plot of the sensory analysis performed on beers. a) odour; b) taste and flavour.
Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.
P value: *, P < 0.05; **, P < 0,01; ***, P < 0.001; not significant values are not reported in figure.

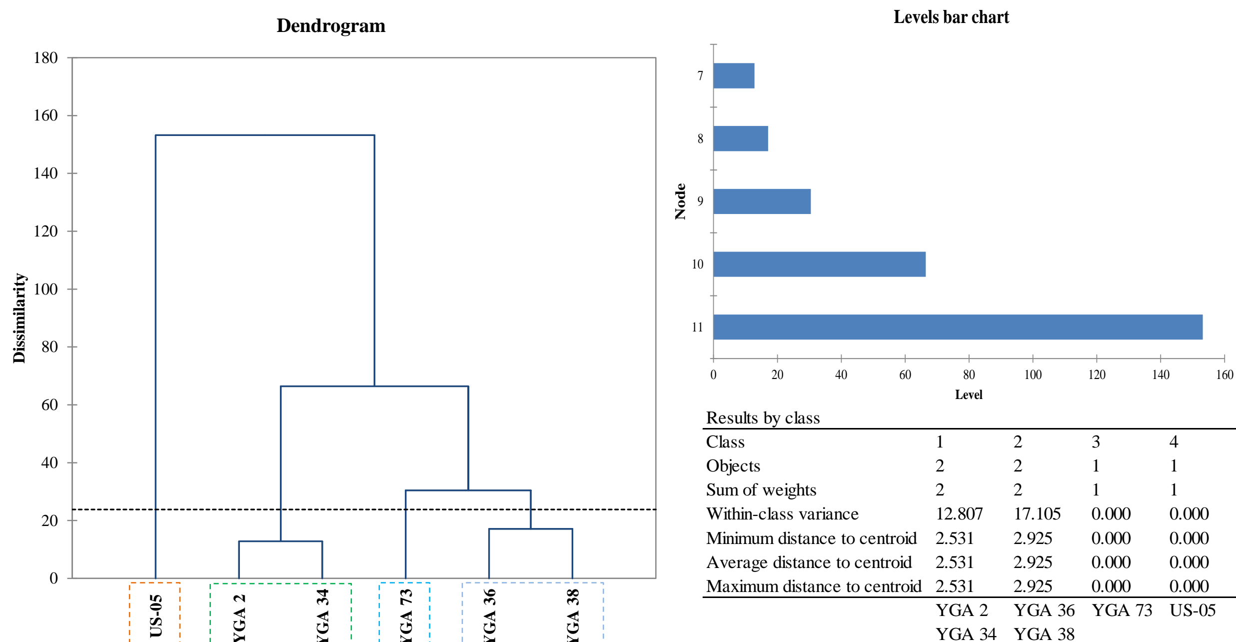
None of the experimental beers showed off-odours and/or off-flavours. No differences were found in terms of colour appearance. The highest score for overall quality was found for beers of trials T2 and T3. Beers fermented in presence of *H. uvarum* YGA34 strain, especially trials T1 and T3, showed highest score for intensity, complexity, floral descriptors in odorous profile as well as for intensity, complexity, sour, sapidity in taste-flavour profile. Sensory differences were also recognized within the trials fermented with YGA34 strain; the trial T3, which involved the inoculum of *H. uvarum* 48 h before that of *S. cerevisiae*, showed the highest value of sour and sapidity descriptors in the taste profile, whereas trial T2 showed the lowest values for the same attributes.

2.3.6. Statistical and explorative multivariate analyses

The Barlett's sphericity test was applied to all data matrix inputs and differences statistically ($p < 0.0001$) significant were found among trials.

AHC analysis discriminated all technological variables into four clusters (classes).

The variance between classes was significantly higher (73.29%) than that found within classes (26.71%). Dendrogram and bar chart (Figure 7) show a deeper analysis of classes by focusing on dissimilarity (%) found among strains (objects). The strains YGA2, YGA34 and YGA36, YGA38 clustered into two classes with values of within-class variance lower than 12.81% and 17.10%, respectively. The strain YGA73 significantly differed from the other *H. uvarum* strains; the control strain *S. cerevisiae* US-05 was out of groups and reached values of dissimilarity higher than 150.



Results by class

Class	1	2	3	4
Objects	2	2	1	1
Sum of weights	2	2	1	1
Within-class variance	12.807	17.105	0.000	0.000
Minimum distance to centroid	2.531	2.925	0.000	0.000
Average distance to centroid	2.531	2.925	0.000	0.000
Maximum distance to centroid	2.531	2.925	0.000	0.000
	YGA 2 YGA 34	YGA 36 YGA 38	YGA 73	US-05

Figure 7. Dendrogram of strains per each class resulting from AHC analysis based on values of technological screening of *H. uvarum* strains Dissimilarity is calculated by Euclidean distance and Ward's agglomeration method. The analysis is based on values of technological variables reported at paragraph 2.5. Codes: YGA2, YGA34, YGA36, YGA38 and YGA73 refer to *H. uvarum* strains; US-05refers to *S. cerevisiae* commercial strain.

PCA was used to condense all technological information into a reduced number of Factors. The results of PCA showed that all five eigen-values were higher than 1. Even though, Factor 1 and Factor 2 explained very high values (58.08 and 23.92%, respectively) of total variance; the classes represented by strains the YGA36, YGA38 and YGA73 were not statistically discriminated. A deeper differentiation among strains was achieved by PCA based on Factor 2 and Factor 3 accounting for a 23.92% and 10.91% of total variability, respectively.

The components of PCA were correlated to variables as shown in the Figures 8A and 8B. By biplot analysis based on F1 and F2 (Fig. 8A) the strains YGA2 and YGA34 were closely related to the best growth in presence of ethanol (5% v/v) and high concentration of hop (50 and 90 IBU). Even though, the components F2 and F3 contributed to differentiate strain YGA73 from the strains YGA36 and YGA38, all these strains were correlated to the highest values of lag phase and maximum growth in presence of ethanol and hop (Fig. 8A and 8B). The control strain US-05 was clearly discriminated from *H. uvarum* strains.

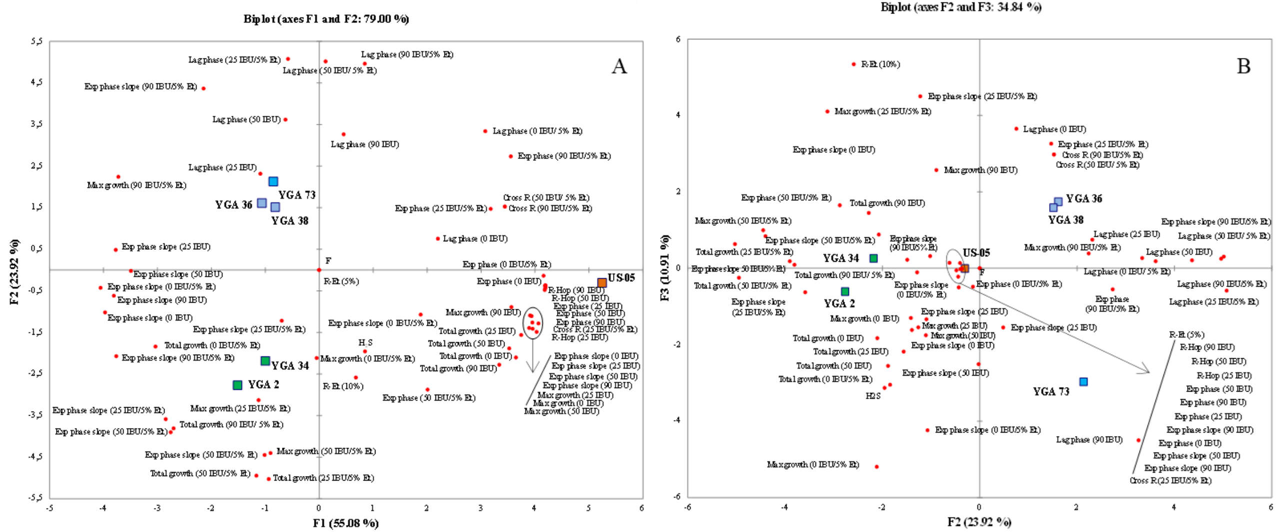


Figure 8. Biplot graph of PCA based on the values of technological screening of *H. uvarum* strains. The analysis is based on values of technological variables reported at paragraph 2.5. Abbreviations: Exp, exponential; R, resistance; F, flocculation; Et, ethanol; IBU, international bitter units that refer to hope concentration. Codes: YGA2, YGA34, YGA36, YGA38 and YGA73 refer to *H. uvarum* strains; US-05 refers to *S. cerevisiae* commercial strain.

Further insights on correlation between strains and technological variables were provided by HMC analysis (Figure 9).

This analysis was performed to obtain a deeper strain differentiation based on the technological variable values. Since a total of 58 variables were analysed, the HMC colour intensity clearly showed the main differences among strains.

Regarding statistical multivariate analysis on VOCs, the double hierarchical dendrogram combined with heat map plot (Figure 5) showed that all trials significantly affected the VOCs composition of the beers. Interestingly, control trial fermented with *S. cerevisiae* US-05 strain Tc resulted in a separate single cluster, while the trials performed with *H. uvarum* inoculums grouped into a different branch, with T1 and T2 clustering together and trial T3 forming a separate single cluster. Thus, the biplot in Figure 10 represents the final distribution of trials with respect to the different VOCs detected. In particular, trial T3 was mostly affected by ethyl acetate, isoamyl acetate and hexanal variables.

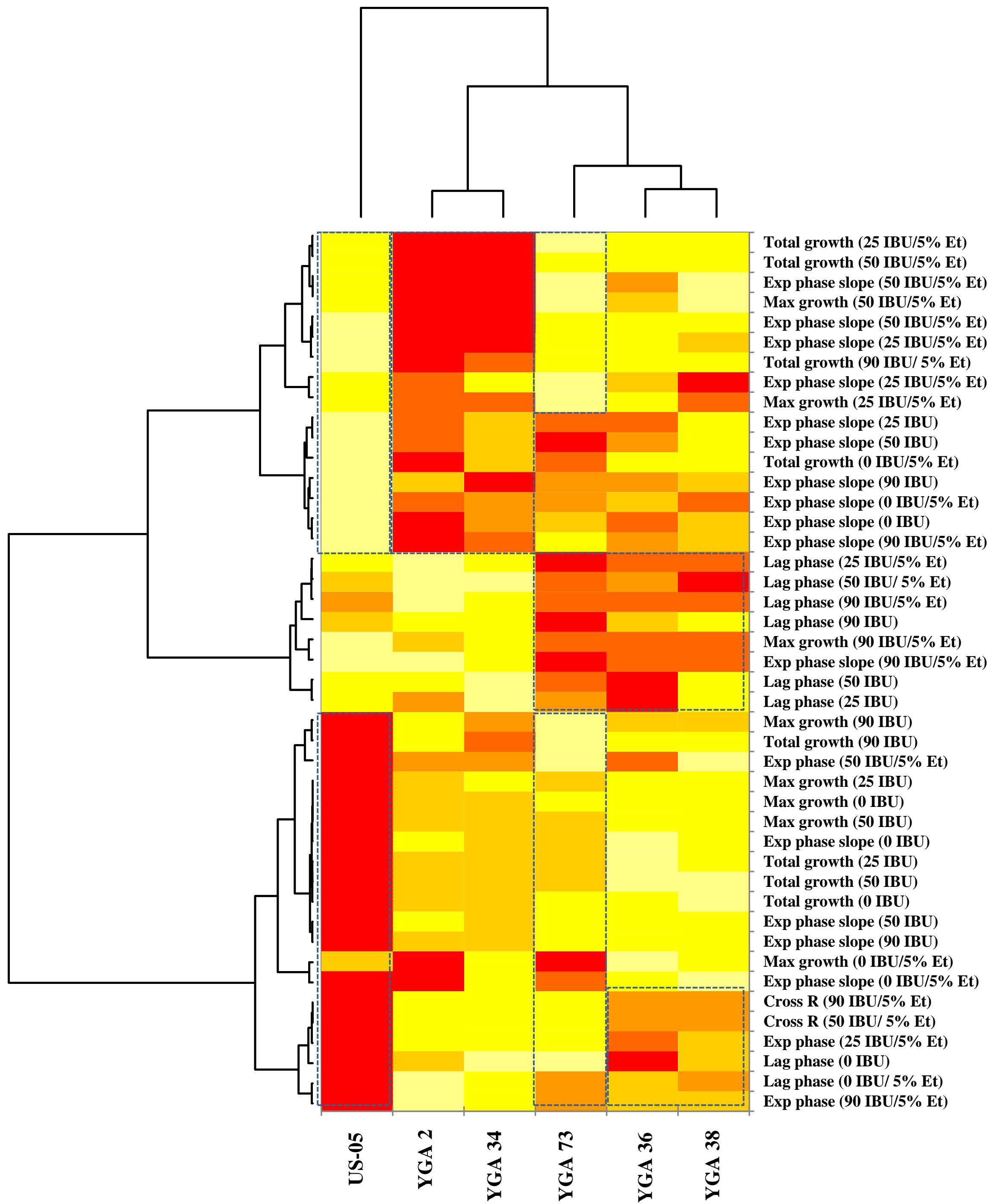


Figure 9. Distribution of technological variables among strains. The heat map plot depicts the relative percentage of values per each technological variable (Y-axis clustering) within each strain (X-axis clustering). The analysis is based on values of technological variables reported at paragraph 2.5. Codes: YGA2, YGA34, YGA36, YGA38 and YGA73 refer to *H. uvarum* strains; US-05 refers to *S. cerevisiae* commercial strain.

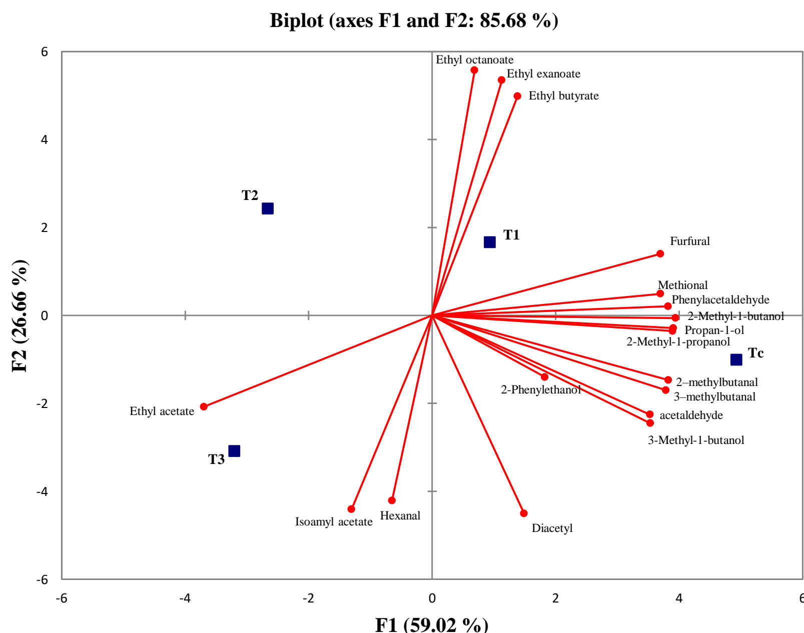


Figure 10. PCA based on the values of VOCs. Biplot graphs show relationships among factors, variables and trials. Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

The statistical multivariate investigation was carried out also on data from sensory analysis. Due to high numbers of sensory attributes and trials, the HCA was applied to identify significant differences among trials. Interestingly, control trial clustered together with trial T2, while T1 and T3 trials formed single clusters. The biplot reported in Figure 11 and heat-map in Figure 12 represent the final distribution of all experimental trials and the control.

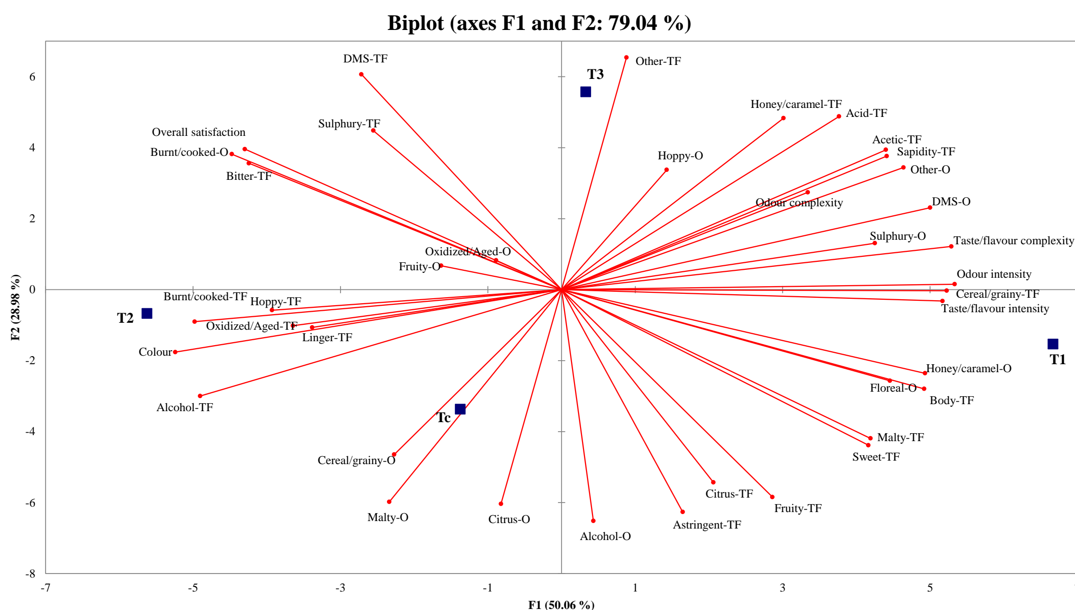


Figure 11. PCA for sensory data of beers. Biplot graphs show relationships among factors, variables and trials. Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

The trials T1 and T3 produced the best beers in terms of odour intensity and complexity, and acetic, sapidity and acid notes for the taste profile. Trial T1 was characterized by high values of floral, honey/caramel, malty, sweet and body aroma level. On the other hand, the trials T2 and Tc were mostly influenced by the parameters of alcohol and burnt/cooked, with a high persistency of taste.

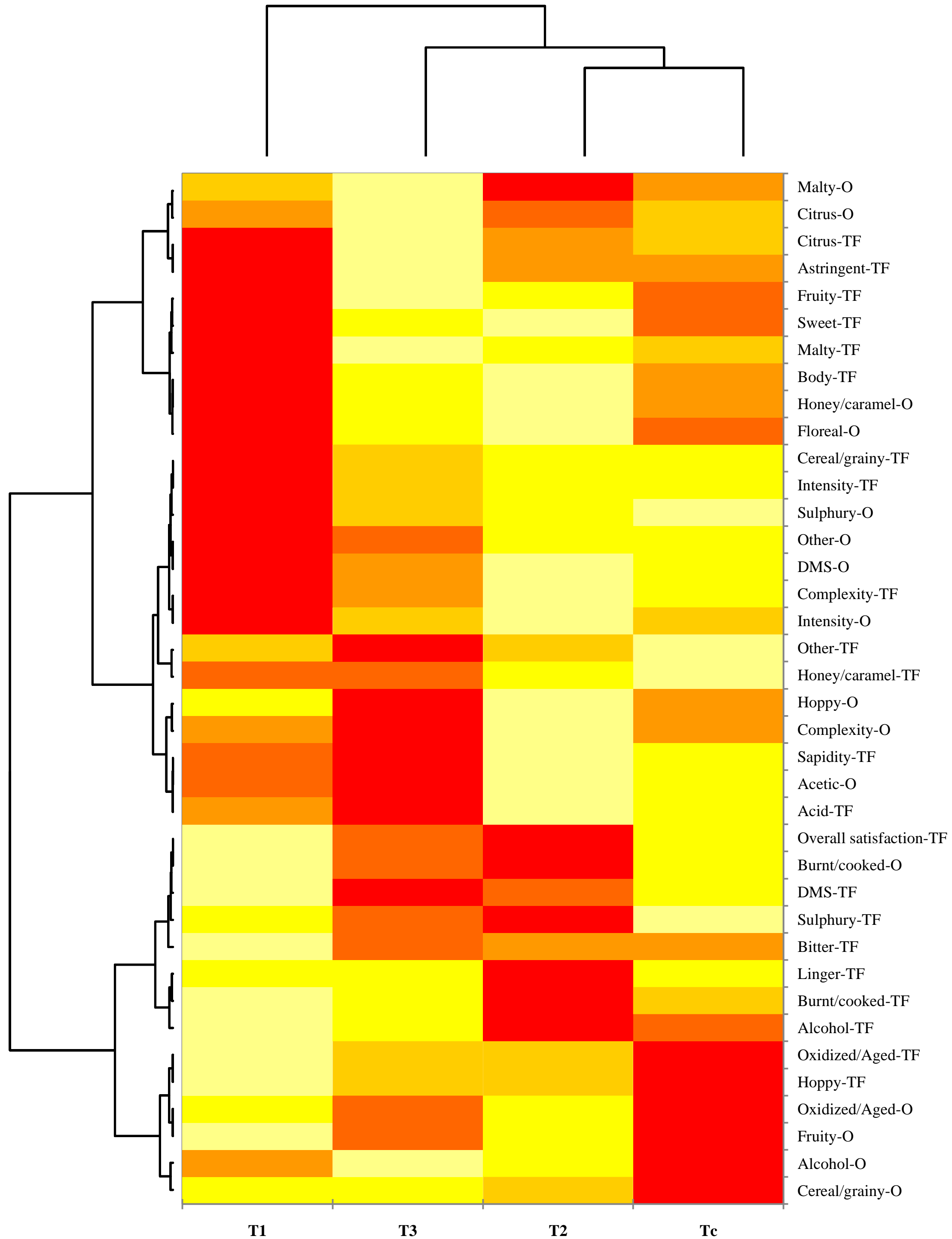


Figure 12. Distribution of sensory descriptors among experimental beers. The heat map plot depicts the relative score of each aroma, taste and overall satisfaction (variables clustering on the Y-axis) within each trial (X-axis clustering). Codes: T1 refers to co-inoculated fermentation with 1:1 ratio between YGA34 and US-05; T2 refers to co-inoculated fermentation with 10:1 ratio between YGA34 and US-05; T3 refers to sequentially inoculated fermentation with YGA34 and US-05; Tc refers to fermentation trial inoculated with monoculture of US-05.

2.4. DISCUSSION

The present research has two main focuses: to characterize the yeast ecology associated to honey by-products subjected to the spontaneous AF; and to select novel non-*Saccharomyces* strains to be used as co-starter culture for craft beer production.

The quality of beer depends on several ingredients, such as malt, hop, water. Moreover, the yeasts used as fermenting starters are of paramount relevance to improve shelf life and sensory characteristics of beer. The majority of beers are fermented by using strains of *S. cerevisiae* and *S. pastorianus* species commercially available worldwide. Recently, non-conventional yeasts gained popularity among brewers in order to obtain distinctive products thanks to the diversity of substrate assimilation patterns that may be displayed. Non-conventional yeasts in brewing are represented by *Saccharomyces* and non-*Saccharomyces* yeasts isolated from non-brewing environment (Catallo et al., 2020; Cubillos et al., 2019). With these regards, Cubillos et al. (2019) published an updated review pertaining to the use of wild *Saccharomyces* and non-*Saccharomyces* in brewing system. Those authors focused the research on the diversity of spontaneously fermented food systems (wine, fruit, dough), as well as on fermented sugary matrixes (cachaça, kombucha) (Araújo et al., 2018; Bellut et al., 2018; Gutiérrez et al., 2018; van Rijswijck et al., 2017), showing that several matrices are promising for yeast isolation for brewing application.

With regards to unconventional yeasts, the use of non-*Saccharomyces* strains in controlled fermentations is a key factor to gain market share (Ciani and Comitini, 2011; Cordero-Bueso et al., 2013; González et al., 2013; Johnson, 2013; van Dijken, 2002). Different strains of *Torulaspora delbrueckii* isolated from different matrices including wine, soil, fruits, sugar cane, papaya leaves, sugar cane juice, grapes, fig fruit, coconut palm and corossol fruit have been screened and applied for beer production (Canonico et al., 2016). Furthermore, *Zygosaccharomyces rouxii* was used by De Francesco et al. (2015) as starter culture for low-alcohol beer production and *Brettanomyces bruxellensis*, isolated from fruit by Lentz et al. (2014), was tested for the ability to ferment wort sugars and for ethanol tolerance. Then, it is now widely accepted that traditional fermented beverages and non-conventional yeasts represent a novelty for brewers (Cubillos et al., 2019).

Up to date, the use of honey in beer industry has been only related to the addition as ingredients for wort and/or green beer production, or as food adjuvant to improve sensory profile of bottled beer (Lu et al., 2011).

With this in mind, the present paper focused on yeast ecology of fermented honey niches as novel sources for non-*Saccharomyces* yeasts. Sinacori et al. (2014) find out that *Zygosaccharomyces mellis* and *Zygosaccharomyces rouxii* were isolated from different samples of honey from different geographical and botanical origins. Gaglio et al. (2017) highlighted the presence of different species

of non-*Saccharomyces* including *Lachancea fermentati*, *Wickerhamomyces anomalus* and *Zygosaccharomyces* spp., mainly. In the present study, contrarily to the works of Sinacori et al. (2014) and Gaglio et al. (2017), yeast ecology of FHP was mainly represented by isolates ascribable to *H. uvarum*. Actually, the role of this species in brewing system is still uninvestigated. As widely reported in scientific literature, *H. uvarum* is mostly associated to the winery industry. This species is associated with the grape surface and is characterized by the ability to assimilate glucose rapidly (Pretorius, 2000) and for its high production of β-glycosidase (Arévalo Villena et al., 2005; Fia et al., 2005). Several species within *Hanseniaspora* genus have been recently investigated for their ability to improve aroma profile of wine, cider and cachaça (Grijalva-Vallejos et al., 2020).

Regarding to the use of *Hanseniaspora* spp. strains in brewing, so far very limited data have been published. To our knowledge, a few *Hanseniaspora* spp. were isolated from spontaneously fermented Lambic beer (Spitaels et al., 2014), but their role still remains unrevealed. The species *Hanseniaspora valbyensis* and *Hanseniaspora vineae* were screened to evaluate their application for the production of alcohol-free beer (Bellut et al., 2018), and *H. vineae* strain YH72, isolated from ash bark was tested for the ability to acidify wort in sour beer production (Osburn et al., 2018). The description of *Hanseniaspora gamundiae* from sugar-rich fungal stromata of *Cyttaria hariotii* highlights the importance of matrices containing high concentrations of carbohydrates to isolate yeasts to be tested in beverage fermentations (Cadez et al., 2019).

Focusing on the technological performances of the five strains of *H. uvarum* of our study, an interesting tolerance to ethanol was observed. Except strain YGA73, all other strains showed growth on medium containing 10% (v/v) ethanol, even though weak. Several species of non-*Saccharomyces* yeasts are not able to grow at this ethanol concentration. In literature it is reported that non-*Saccharomyces* yeasts like *Hanseniaspora* spp. are known as low resistant to ethanol. *Hanseniaspora vinae* (Bellut et al., 2018; Benito et al., 2019; Osburn et al., 2018), for example, does not tolerate ethanol concentrations higher than 4% (v/v), although this trait is not genus or species dependent but strain-dependent (Martin et al., 2018).

Based on these considerations, *H. uvarum* strains selected in this study might be applied as co-starters for producing beer up to 10% (v/v) ethanol. Regarding the cross hop/ethanol resistance, our strains showed a remarkable growth kinetics in presence of high concentration of iso-α-acids and ethanol concentrations. The five strains showed growth phases better than the commercial *S. cerevisiae* strain US-05 used as positive control. These findings indicated that the tested strains possess useful characteristics to be used as co-starters for the production of a wide range of beer worts, including highly hopped worts. The results of FR showed a very low ability of *H. uvarum* strains to drive an AF in beer wort, but plate counts demonstrated the presence of all strains at high levels (7.5-8.2 Log

CFU/ml) up to 12 d of wort fermentation. The low FR does not represent a limitation for beer production; several species of non-*Saccharomyces* are characterized by very low fermentation power and/or vigour, but most of them are able to improve the sensory characteristics of fermented beverages such as beer and wine. For example, the strain *Metschnikowia pulcherrima* Flavia® (Lallemand Inc., Montreal, Canada), does not ferment grape sugars into ethanol but, it is one the most largely used strains in winemaking as co-starter due to its intense production of β-glucosidase which changes the wine aromatic profile by increasing smoky and flowery notes (Rodriguez et al., 2007, 2010; Ruiz et al., 2018; Sadineni et al., 2012; Su et al., 2020). Even *Hanseniaspora/Kloeckera* yeast could affect the wine fermentation results, by modelling flavour profile and the metabolism of *S. cerevisiae* commercial strains, due different secondary metabolic pathways and an increased enzymatic activity (Martin et al., 2018).

Multivariate data analysis has been widely applied in food processes (Berrueta et al., 2007) and, recently, extensively applied in beer research (Snauwaert et al., 2016). In our study, an extensive data analysis based on 58 technological variables was performed. Both PCA and HMC analyses were able to find the significant correlation among strains, variable and values confirming their usefulness to underline the technological properties of yeast strains.

Even though in trial T1 a dominance of *S. cerevisiae* populations was registered after 48 h, *H. uvarum* developed at higher cell densities than *S. cerevisiae* in trial T2. When the inoculums of *H. uvarum* and *S. cerevisiae* occurred sequentially (trial T3), the dominance of *H. uvarum* persisted throughout the fermentation process, with the populations of *S. cerevisiae* being below 6.7 Log CFU/mL. In trial T4, despite the inability of *H. uvarum* to consume the complex sugars of the wort, its levels were close to 7.5 Log CFU/ml.

Actually, it has been not reported maltose fermentation for the *Hanseniaspora* genus and genes for maltose assimilation were not identified in public available genomes of *Hanseniaspora* spp. (Cadez and Smith, 2011; Cadez et al., 2019).

The strain *H. uvarum* YGA34 was able to produce considerable amounts of acetic acid, although at lower concentrations than the control trial, already during the first 48 h of AF. Regarding glycerol production, the trial showing the highest amounts was that involving the sequential inoculation, while no substantial differences among the trials were detected for ethanol production.

Despite the metabolic inability of *H. uvarum* YGA34 strain to assimilate and ferment maltose, the effect of its application during the earlier stage of beer fermentation was highlighted by sensory and VOC analyses.

The most abundant VOC category was alcohols that are known for enhancing flowery, solvent like or alcoholic flavours, however their presence above certain threshold could be undesired (Eßlinger,

2009). The second most abundant VOC class has been represented by esters, whose level depend on the yeast strain-specific activity of synthesis and breakdown enzymes (Pires et al., 2014). The main aroma-active ester was ethyl acetate, a secondary metabolite of alcoholic fermentation, responsible of fruity aroma of beers and whose perception threshold is equal to 25 mg/L. Ethyl hexanoate and isoamyl acetate, which are liable for banana, apple and anise aroma, showed values below the perception threshold (Meilgaard, 1975).

Thus, the use of this unconventional strain for beer production led to a novel product mainly under sensory profiles. *H. uvarum* YGA34 showed a certain degree of dominance also in combination with *S. cerevisiae*. The three different inoculation methods highlighted a good co-existence between the two yeast species that generated three different final products, all without any detectable defect. The organoleptic quality of the experimental beers fermented with *H. uvarum* YGA34 were confirmed by sensory analysis that showed high scores for taste complexity and intensity, aroma intensity, acid, sapid descriptors, as well as overall satisfaction.

2.5. CONCLUSION

In conclusion, for the first time, the yeast diversity of FHP, an ancient honey-based beverage produced in Sicily (Italy) was explored for the selection of food starters and provided scientific data on the technological relevance of yeasts from honey and/or FHP for brewing application. This work enriches the very limited scientific knowledge on the role of *Hanseniaspora* yeasts as potential co-starter for beer production. Throughout a genotypic and phenotypic polyphasic approach it was possible to identify and characterize five *H. uvarum* strains. For the first time a high resistance to ethanol and hop in beer wort has been reported for *H. uvarum* strains. The application of these strains during brewing showed differences in terms of physico-chemical parameters, VOCs and sensory traits indicating that *H. uvarum* strains are promising as co-starter in a wide range of beer productions. However, further investigations are needed to evaluate the role of these strains during wort fermentation in presence of different strains of *S. cerevisiae* and with different beer wort composition.

2.6. REFERENCES

Ajovalasit, L., Columba, P., 1998. Prodotti tipici da scoprire: *Spiritu re fascitrari*. Econ. Agro-Alim. 1, 1-9.

Araújo, T.M., Souza, M.T., Diniz, R.H.S., Yamakawa, C.K., Soares, L.B., Lenczak, J.L., Oliveira, J.V.D.C., Goldman, G.H., Barbosa, E.A., Campos, A.C.S., Brandão R.L., Castro, I.M., 2018. Cachaça yeast strains: alternative starters to produce beer and bioethanol. Antonie Van Leeuwenhoek 111(10), 1749-1766.

Arévalo Villena, M., Ubeda Iranzo, J.F, Cordero Otero, R.R, Briones Perez, A.I., 2005. Optimization of a rapid method for studying the cellular location of *β*-glucosidase activity in wine yeasts. J. Appl. Microbiol. 99, 558-64.

Barquet, M., Martín, V., Medina, K., Pérez, G., Carrau, F., Gaggero, C., 2012. Tandem repeat-tRNA (TRtRNA) PCR method for the molecular typing of non-*Saccharomyces* subspecies. Applied microbiology and biotechnology, 93(2), 807-814.

Bellut, K., Michel, M., Zarnkow, M., Hutzler, M., Jacob, F., De Schutter, D.P., Daenen, L., Lynch, K.M., Zannini, E., Arendt, E.K., 2018. Application of non-*Saccharomyces* yeasts isolated from Kombucha in the production of alcohol-free beer. Fermentation 4(3), 66.

Benito, Á., Calderón, F., Benito, S., 2019. The Influence of Non-*Saccharomyces* Species on Wine Fermentation Quality Parameters. Fermentation 5(3), 54.

Berrueta, L.A., Alonso-Salces, R.M., Héberger, K., 2007. Supervised pattern recognition in food analysis. J. Chromatogr. A 1158(1-2), 196-214.

Bokulich, N.A., Bamforth, C.W., Mills, D.A., 2012. Brewhouse-resident microbiota are responsible for multi-stage fermentation of American coolship ale. PloS ONE 7(4), e35507.

Cadez, N., Bellora, N., Ulloa, R., Hittinger, C.T., Libkind, D., 2019. Genomic content of a novel yeast species *Hanseniaspora gamundiae* sp. nov. from fungal stromata (*Cyttaria*) associated with a unique fermented beverage in Andean Patagonia, Argentina. PLoS ONE 14(1), e0210792.

Cadez, N., Pagnocca, F.C., Raspor, P., Rosa, C.A., 2014. *Hanseniaspora nectarophila* sp. nov., a yeast species isolated from ephemeral flowers Int. J. Syst. Evol. Microbiol. 64(7), 2364-2369.

Cadez, N., Smith, M.T., 2011. *Hanseniaspora* Zikes (1912). In: Kurtzman, C., Fell, J.W., Boekhout, T. (Eds.), Yeasts A Taxon. Study.

Canonico, L., Agarbati, A., Comitini, F., Ciani, M., 2016. *Torulaspora delbrueckii* in the brewing process: A new approach to enhance bioflavour and to reduce ethanol content. Food Microbiol. 56, 45-51.

Capece, A, Fiore, C, Maraz, A, Romano, P., 2005. Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. J Appl Microbiol 98, 136-144.

Casey, G., van der Aar, P., Barbero, F., Bendiak, D., DeBruyn, L., D'Amore, T., Gallegos, D., Gonzalez, M., Hekel, J., O'Connell, J., Pugh, T., Sobczak, J., Sondag, R., Speer, A., Wright, G., Yabuuchi, S., Crumplen, R., 1994. Yeast flocculation determination by the Helm assay. J. Am. Soc. Brew. Chem. 52:4, 188-191.

Catallo, M., Nikulin, J., Johansson, L., Krogerus, K., Laitinen, M., Magalhães, F., Piironen, M., Mikkelson, A., Randazzo, C.L., Solieri, L., Gibson, B., 2020. Sourdough derived strains of *Saccharomyces cerevisiae* and their potential for farmhouse ale brewing. J. Inst. Brew., 126(2), 168-175.

Ciani, M., Comitini, F., 2011. Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. Ann. Microbiol. 61(1), 25-32.

Ciani, M., Maccarelli, F., 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World J. Microbiol. Biotechnol. 14(2), 199-203.

Cordero-Bueso, G., Esteve-Zarzoso, B., Cabellos, J.M., Gil-Díaz, M., Arroyo, T., 2013. Biotechnological potential of non-*Saccharomyces* yeasts isolated during spontaneous fermentations of Malvar (*Vitis vinifera* cv. L.). Eur. Food Res. Technol. 236(1), 193-207.

Cubillos, F.A., Gibson, B., Grijalva-Vallejos, N., Krogerus, K., Nikulin, J., 2019. Bioprospecting for brewers: Exploiting natural diversity for naturally diverse beers. Yeast 36(6), 383-398.

De Francesco, G., Turchetti, B., Sileoni, V., Marconi, O., Perretti, G., 2015. Screening of new strains of *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* to produce low- alcohol beer. J. Inst. Brew. 121(1), 113-121.

Dillon, W.R., Goldstein, M., 1984. Multivariate Analysis: Methods and Applications. John Wiley, New York.

Eßlinger, H. M., 2009. Handbook of brewing: processes, technology, markets. John Wiley & Sons.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Evol. Microbiol. 49(1), 329-337.

Fia, G., Giovani, G., Rosi, I., 2005. Study of *β*-glucosidase production by wine-related yeasts during alcoholic fermentation: a new rapid fluorimetric method to determine enzymatic activity. J. Appl. Microbiol. 99, 509-17.

Francesca, N., Carvalho, C., Sannino, C., Guerreiro, M.A., Almeida, P.M., Settanni, L., Massa B., Sampaio, J.P., Moschetti, G., 2014. Yeasts vectored by migratory birds collected in the Mediterranean island of Ustica and description of *Phaffomyces usticensis* fa sp. nov., a new species related to the cactus ecoclade. FEMS Yeast Res. 14(6), 910-921.

Francesca, N., Chiurazzi, M., Romano, R., Aponte, M., Settanni, L., Moschetti, G., 2010. Indigenous yeast communities in the environment of "Rovello bianco" grape variety and their use in commercial white wine fermentation. World J. Microbiol. Biotechnol. 26(2), 337-351.

Gaglio, R., Alfonzo, A., Francesca, N., Corona, O., Di Gerlando, R., Columba, P., Moschetti, G., 2017. Production of the Sicilian distillate "*Spiritu re fascitrari*" from honey by-products: An interesting source of yeast diversity. Int. J. Food Microbiol. 261, 62-72.

Glover, B., 2001. The World Encyclopedia of Beer. Lorenz Books, Anness Publishing limited, New York.

González, R., Quirós, M., Morales, P., 2013. Yeast respiration of sugars by non-*Saccharomyces* yeast species: a promising and barely explored approach to lowering alcohol content of wines. Trends Food Sci. Technol. 29(1), 55-61.

Grijalva-Vallejos, N., Aranda, A., Matallana, E., 2020. Evaluation of yeasts from Ecuadorian chicha by their performance as starters for alcoholic fermentations in the food industry. Int. J. Food Microbiol. 317, 108462.

Gutiérrez, A., Boekhout, T., Gojkovic, Z., Katz, M., 2018. Evaluation of non-*Saccharomyces* yeasts in the fermentation of wine, beer and cider for the development of new beverages. J. Inst. Brew. 124(4), 389-402.

Hall, B.G., Acar, H., Nandipati, A., Barlow, M., 2014. Growth rates made easy. Mol. Biol. Evol. 31(1), 232-238.

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K.J., Thevelein, J.M., 2018. Bioflavoring by non-conventional yeasts in sequential beer fermentations. Food Microbiol. 72, 55-66.

Issa-Issa, H., Guclu, G., Noguera-Artiaga, L., López-Lluch, D., Poveda, R., Kelebek, H., Selli, S., Carbonell-Barrachina, Á.A., 2020. Aroma-active compounds, sensory profile, and phenolic composition of Fondillón. Food Chemistry, 316, 126353.

Jackson, R.S. 2016. Wine tasting. A professional handbook, third edition. Eds. Cool Climate enology and Viticulture Institute, Brock University, St. Catharines, Ontario, Canada.

Jindamorakot, S., Ninomiya, S., Limtong, S., Yongmanitchai, W., Tuntirungkij, M., Potacharoen, W., Tanaka, K., Kawasaki., H., Nakase, T., 2009. Three new species of bipolar budding yeasts of the genus *Hanseniaspora* and its anamorph *Kloeckera* isolated in Thailand. FEMS Yeast Res. 9(8), 1327-1337.

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. Appl. Environ. Microbiol. 61(2), 461-467.

Johnson, E.A., 2013. Biotechnology of non-*Saccharomyces* yeasts - the ascomycetes. Applied Microbiol. Biotechnol. 97(2), 503-517.

Katz, S.H., Maytag, F., 1991. Brewing an ancient beer. Archaeology 44(4), 24-27.

Kurtzman, C.P., Fell, J.W., Boekhout, T., Robert, V., 2011. Methods for isolation, phenotypic characterization and maintenance of yeasts. In: Kurtzman, C.P., Fell, J.W., Boekhout, T.(Eds.), The Yeasts: A Taxonomic Study, Fifth ed. Elsevier, USA, pp. 87-110.

Lappe-Oliveras, P., Moreno-Terrazas, R., Arrizón-Gaviño, J., Herrera-Suárez, T., García-Mendoza, A., Gschaedler-Mathis, A., 2008. Yeasts associated with the production of Mexican alcoholic nondistilled and distilled Agave beverages. *FEMS yeast research*, *8*(7), 1037-1052.

Larroque, M.N., Carrau, F., Fariña, L., Boido, E., Dellacassa, E., Medina, K., 2021. Effect of *Saccharomyces* and non-*Saccharomyces* native yeasts on beer aroma compounds. Int. J. Food Microbiol. 337, 108953.

Legras, J., Karst, F., 2003. Optimisation of interdelta for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiol. Lett. 221, 249-255.

Lentz, M., Putzke, T., Hessler, R., Luman, E., 2014. Genetic and physiological characterization of yeast isolated from ripe fruit and analysis of fermentation and brewing potential. J. Inst. Brew. 120, 559-564.

Lu, Z.F., Li, J.Y., Li, C., 2011. Development of Honey Beer [J]. Liquor-Making Science & Technology, 4.

Malfliet, S., Van Opstaele, F., De Clippeleer, J., Syryn, E., Goiris, K., DeCooman, L., and Aerts G. 2009. Flavour instability of pale lager beers: Determination of analytical markers in relation to sensoryageing, J. Inst. Brew. 114, 180-192.

Marconi, O., Rossi, S., Galgano, F., Sileoni, V., Perretti, G., 2016. Influence of yeast strain, priming solution and temperature on beer bottle conditioning. J. Sci. Food Agric. 96(12), 4106-4115.

Marongiu, A., Zara, G., Legras, J.L., Del Caro, A., Mascia, I., Fadda, C., Budroni, M., 2015. Novel starters for old processes: use of *Saccharomyces cerevisiae* strains isolated from artisanal sourdough for craft beer production at a brewery scale. J. Ind. Microbiol. Biotechnol. 42(1), 85-92.

Martin, V., Valera, M. J., Medina, K., Boido, E., Carrau, F., 2018. Oenological impact of the *Hanseniaspora/Kloeckera* yeast genus on wines - a review. *Fermentation*, *4*(3), 76.

Martorana, A., Alfonzo, A., Settanni, L., Corona, O., La Croce, F., Caruso, T., Moschetti, G., Francesca, N., 2015. An innovative method to produce green table olives based on “*pied de cuve*” technology. Food Microbiol. 50, 126-140.

Mazzei, P., Francesca, N., Moschetti, G., Piccolo, A., 2010. NMR spectroscopy evaluation of direct relationship between soils and molecular composition of red wines from Aglianico grapes. Anal. Chim. Acta 673(2), 167-172.

Mazzei, P., Spaccini, R., Francesca, N., Moschetti, G., Piccolo, A., 2013. Metabolomic by ^{1}H NMR spectroscopy differentiates "Fiano Di Avellino" white wines obtained with different yeast strains. J. Agric. Food Chem. 61(45), 10816-10822.

Meilgaard, M.C., 1975. Flavor chemistry of beer. Part II. Flavor and threshold of 239 aroma volatiles. Tech. Quart. Master. Brew. Assoc. Am., 12, 151-168.

Michel, M., Kopecká, J., Meier-Dörnberg, T., Zarnkow, M., Jacob, F., Hutzler, M., 2016. Screening for new brewing yeasts in the non-*Saccharomyces* sector with *Torulaspora delbrueckii* as model. Yeast 33(4), 129-144.

Nikulin, J., Eerikäinen, R., Hutzler, M., Gibson, B., 2020. Brewing Characteristics of the Maltotriose-Positive Yeast *Zygotorulaspora florentina* Isolated from Oak. *Beverages*, *6*(4), 58.

Osburn, K., Amaral, J., Metcalf, S.R., Nickens D.M., Rogers, C.M., Sausen, C., Caputo, R., Miller, J., Li, H., Tennessen, J.M., Bochman, M.L., 2018. Primary souring: A novel bacteria-free method for sour beer production. Food Microbiol. 70, 76-84.

Pires, E.J., Teixeira, J.A., Brányik, T., Vicente, A.A., 2014. Yeast: the soul of beer's aroma - a review of flavour-active esters and higher alcohols produced by the brewing yeast. Appl. Microbiol. Biotechnol. 98(5), 1937-1949.

Pretorius, I.S., 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16(8), 675-729.

Protz, R., 1995. The ultimate encyclopedia of beer: the definitive guide to the world's great brews, first ed. Carlton.

Rodríguez, M.E., Lopes, C., Valles, S., Giraudo, M.R., Caballero, A., 2007. Selection and preliminary characterization of *β*-glycosidases producer Patagonian wild yeasts. Enzyme Microb. Technol. 41(6-7), 812-820.

Rodríguez, M.E., Lopes, C.A., Barbagelata, R.J., Barda, N.B., Caballero, A.C., 2010. Influence of *Candida pulcherrima* Patagonian strain on alcoholic fermentation behaviour and wine aroma. Int. J. Food Microbiol. 138(1-2), 19-25.

Ruiz, J., Belda, I., Beisert, B., Navascués, E., Marquina, D., Calderón, F., Rauhut, D., Santos, A., Benito, S., 2018. Analytical impact of *Metschnikowia pulcherrima* in the volatile profile of Verdejo white wines. Appl. Microbiol. Biotechnol. 102(19), 8501-8509.

Sadineni, V., Kondapalli, N., Obulam, V.S.R., 2012. Effect of co-fermentation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* or *Metschnikowia pulcherrima* on the aroma and sensory properties of mango wine. Ann. Microbiol. 62(4), 1353-1360.

Salvadó, Z., Arroyo-López, F.N., Guillamón, J.M., Salazar, G., Querol, A., Barrio, E., 2011. Temperature adaptation markedly determines evolution within the genus *Saccharomyces*. Appl. Environ. Microbiol. 77(7), 2292-2302.

Sannino, C., Francesca, N., Corona, C., Settanni, L., Cruciata, M., Moschetti, G., 2013. Effect of the natural winemaking process applied at industrial level on the microbiological and chemical characteristics of wine. J. Biosci. Bioeng. 116, 347-356.

Sannino, C., Mezzasoma, A., Buzzini, P., Turchetti, B., 2019. Non-conventional Yeasts for Producing Alternative Beers. In: Sibirny, A. (Ed.), Non-conventional Yeasts: from Basic Research to Application, Springer, Cham, pp. 361-388.

Sinacori, M., Francesca, N., Alfonzo, A., Cruciata, M., Sannino, C., Settanni, L., Moschetti, G., 2014. Cultivable microorganisms associated with honeys of different geographical and botanical origin. Food Microbiol. 38, 284-294.

Snauwaert, I., Roels, S.P., Van Nieuwerburgh, F., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2016. Microbial diversity and metabolite composition of Belgian red-brown acidic ales. Int. J. Food Microbiol. 221, 1-11.

Spitaels, F., Wieme, A.D., Janssens, M., Aerts, M., Daniel, H.M., Van Landschoot, A., De Vuyst, L., Vandamme, P., 2014. The microbial diversity of traditional spontaneously fermented lambic beer. PLoS ONE 9(4), e95384.

Su, Y., Seguinot, P., Sanchez, I., Ortiz-Julien, A., Heras, J.M., Querol, A., Camarasa, C., Guillamón, J.M., 2020. Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions. Food Microbiol. 85, 103287.

Tofalo, R., Perpetuini, G., Di Gianvito, P., Schirone, M., Corsetti, A., Suzzi, G., 2014. Genetic diversity of FLO1 and FLO5 genes in wine flocculent *Saccharomyces cerevisiae* strains. Int. J. Food Microbiol. 191, 45-52.

van Dijken, H., 2002. The 21st International Specialized Symposium on Yeasts (ISSY 2001) “Biochemistry, Genetics, Biotechnology and Ecology of Non-conventional Yeasts (NCY)”. FEMS Yeast Res. 1, 337-338.

van Rijswijck, I.M., Wolkers-Rooijackers, J.C., Abee, T., Smid, E.J., 2017. Performance of non-conventional yeasts in co-culture with brewers’ yeast for steering ethanol and aroma production. Microb. Biotechnol. 10(6), 1591-1602.

Varela, C., 2016. The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. Appl. Microbiol. Biotechnol. 100(23), 9861-9874.

Vesely, P., Lusk, L., Basarova, G., Seabrooks, J., and Ryder, D., 2003. Analysis of aldehydes in beer using solid-phase microextraction withon-fiber derivatization and gas chromatography/mass spectrometry,J. Agric. Food Chem. 51, 6941–6944.

CHAPTER 3

Technological screening and application of Saccharomyces cerevisiae strains isolated from fermented honey by-products for the sensory improvement of "Spiritu re fascitrari", a typical Sicilian distilled beverage

ABSTRACT

"Spiritu re fascitrari" is a Sicilian alcoholic beverage obtained through distillation of a decoction of spontaneously fermented honey by-products. The production process often leads to sensorial defects due to the unstable alcoholic fermentation. The objective of this work was to select *Saccharomyces cerevisiae* strains from spontaneously fermented honey by-products (FHP) to be used as starter in decoction fermentation. Based on chemical, microbiological and technological data, from a total of 91 strains three *S. cerevisiae* were selected for further testing to produce FHP at laboratory scale level. After FHP distillation, the analysis of volatile organic compounds showed a complex mixture of sensory active molecules, mainly alcohols and aldehydes. Among the alcohols, 3-methyl-1-butanol, 2-methyl-1-butanol, phenylethyl alcohol, hexadecanol and octadecanol were found at the highest concentrations. Among the carboxylic acids, acetic acid was mainly detected in the spontaneously fermented samples. FHP fermented with the three selected strains were not characterized by the presence of off-odours or off-flavours. The results obtained in this work demonstrate that the selected *S. cerevisiae* strains are promising starters to stabilize the production of distilled alcoholic beverages produced from honey by-products.

3.1. INTRODUCTION

Honey production is one of the leading food sectors in Sicily (Osservatorio Nazionale Miele, 2020). Within the characteristic production areas, particularly in Sortino city, honey is recognized as a "traditional food product" and it constitutes a given food chain with other products derived from honey by-products. Among them, in this area, besides honey production, the production of the alcoholic beverage "Spiritu re fascitrari" (SRF) represents a consistent added value product for the beekeepers of the area (Gaglio et al., 2017); it also represents an advantageous strategy to valorise honey production. SRF is similar to mead, but the mass undergoes a distillation process after alcoholic fermentation (AF). However, SRF production is not an easy process; stuck or retarded fermentations often characterize the transformation process of by-products of the traditional processing of honey (honeycombs and capping wax), elongating the fermentation phase even at 6–7 weeks and resulting in off-odour and off-flavour generation. This is imputable to the fact that the transformation process relies exclusively on the spontaneous fermentation carried out by the indigenous yeasts of decoction of the by-products of the processing of honey.

Despite several studies on mead productions have been published, the knowledge on yeast starters is limited and that from honey by-products is even rarer. So far, *Saccharomyces cerevisiae* strains used to ferment mead have been isolated mainly from wine and beer environments (Peepall et al., 2019;

Ramalhosa et al., 2011). In addition to their essential role in AF, yeasts are important for the organoleptic characteristics of mead affecting aroma, flavour and mouthfeel (Peepall et al., 2019). To preserve the quality standards of mead and SRF distillate it is important to reach a balance between sweetness, acidity and alcohols. Recently, novel insights on mead and honey beverages styles have been provided by the American Mead Makers Association (2017) and Beer Judge Certification (2017). As reported by Mead Industry Report (2017), multiple mead styles (spirit barrel aged, oak barrel aged, melomel, cyder, carbonated, metheglin, braggot, and others) have been improved (American Mead Makers Association, 2017).

Honey-must is limited in nitrogen sources necessary for yeast growth (Ramalhosa et al., 2011). The availability of nitrogen can be further reduced in relation to the amount of initial water added to convert raw materials in honey-must (Schwarz et al., 2020). Even though nitrogen nutrients are able to improve the fermentation performance of starter strains (Pereira et al., 2015a, b), the use of selected yeasts with low nitrogen demand is recommended to ferment honey-must. Based on this information, the selection of novel strains with specific characteristics to improve the time of fermentation, the yield in ethanol and the sensory characteristics of bottled products is required.

Therefore, the present study aimed to: (i), differentiate *S. cerevisiae* strains isolated from fermented honey by-products (FHP); (ii), characterize the yeast strains for their main technological traits; (iii), select *S. cerevisiae* strains as potential starter for fermenting honey by-product; and (iv), evaluate the effect of strain inoculum during honey by-product fermentation and sensory quality of distilled products.

3.2. MATERIAL AND METHODS

3.2.1. Yeast cultures, media and reagents

All cultures investigated in the present study have been isolated and identified as *S. cerevisiae* during a survey on honey by-product fermentation and distillation carried out by Gaglio et al. (2017). All cultures were routinely grown on yeast extract, peptone, and dextrose [YPD; 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] plates containing 2% (w/v) agar at 30 °C and in YPD liquid culture at 30 °C in aerobic conditions. All cultures were stored in YPD broth added with 15% (v/v) glycerol at −80 °C and belong to the culture collection of Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo. All media and supplements were purchased from Oxoid (Milan, Italy).

3.2.2. Genotypic differentiation of *S. cerevisiae* isolates

A set of 612 *S. cerevisiae* cultures were subjected to the molecular typing by employing two techniques (Moschetti et al., 2016): interdelta analysis with primers delta12 and delta21 (Legras and Karst, 2003) and microsatellite multiplex PCR based on analysis of the polymorphic microsatellite loci SC8132X, YOR267C and SCPTSY7 (Vaudano and Garcia-Moruno, 2008). PCR products were analysed and visualized as reported by Settanni et al. (2012).

3.2.3. In vitro technological characterization of *S. cerevisiae* strains

All different strains (91) were evaluated for their potential *in vitro* fermentation. The strains were cultured on bismuth sulphite agar (BiGGY Agar, Oxoid, Milan, Italy), Wilson-Blair medium (Merck, Darmstadt, Germany) to evaluate the production of hydrogen sulphide (H_2S; Jiranek et al., 1995). H_2S production was estimated by colony blackening after 3 d of incubation at 28 °C, using a five-level scale as reported by Francesca et al. (2010). *S. cerevisiae* US-05 (Fermentis) and *S. cerevisiae* GR1 (collection of SAAF Department) were used as negative and positive controls, respectively (Araujo et al., 2018; Francesca et al., 2010). The strains characterized by high production levels of acetic acid were identified by the halo produced around the colonies on $CaCO_3$ agar plates after incubation at 25 °C for 7 d. *S. cerevisiae* GR1 and *Hanseniaspora uvarum* TLM14 (SAAF Department culture collection) were used as negative and positive controls, respectively (Settanni et al., 2012). Dilutions of exponential pure cultures were spotted onto Petri dishes containing Malt Extract Agar solid medium added with ethanol concentrations of 12, 14 and 16% (v/v) to perform ethanol tolerance assay (Settanni et al., 2012). The same procedures were used for testing strain growth in presence of 0.15 and 0.25 g/L of potassium metabisulphite (KMBS). Cross resistance of strains to ethanol (12, 14 and 16% v/v) and KMBS (0.15 and 0.25 g/L) was also performed (Settanni et al., 2012). Strain growth was assessed by visual inspection. The growth at low temperatures was determined in YPD broth at 10 °C and 17 °C for 5 d according to the methodology described by Settanni et al. (2012).

3.2.4. Micro-fermentation of mead wort

The strains (14) that showed the best *in vitro* performance were subjected to micro-fermentations in order to determine their fermentation traits.

3.2.4.1. Mead wort preparation

A laboratory-scale fermentation was performed to evaluate the ability of the strains showing the best performances during *in vitro* tests. The procedures of Czabaj et al. (2017) were followed with some modifications to warrant standardized conditions for all trials. The wort fermentation medium was

obtained from multifloral honey (Pagliaro Enterprise, Sortino, Italy) diluted with sterile water in a volumetric proportion to reach a specific gravity of 22 °Bx. The wort was pasteurized at a temperature of 65 °C for 10 min, and, after cooling, aliquots of 4.5 L of wort in 5.0 L flasks and were sealed with a Müller valve to allow the CO_2 produced to escape from the system. One strain was inoculated per each flask; the initial cell density for each inoculated yeast was 10^6 CFU/mL; the fermentations were performed at 10 and 18 °C under static conditions and were monitored daily by measuring the weight loss of CO_2 until it was lower than 0.01 g/day. All fermentations were performed in duplicates.

3.2.4.2. Determination of fermentation parameters

Analytical determinations of fermentation parameters were performed as described by Ciani and Maccarelli (1998). In particular, the following indices were monitored: (i) fermentation rate (FR) was determined as daily CO_2 production after 3 days of wort AF and was expressed in g of CO_2/day; (ii) fermentation vigour (FV) expressed as the amount of ethanol (% v/v) produced by a strain at the end of AF; (iii) fermentation purity (FP) indicating the ratio between volatile acidity and ethanol produced expressed as volatile acidity g/L ethanol % (v/v).

The analyser iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd. Shenzhen, China) was used and run with full automation for the enzymatic assay of ethanol and acetic acid. The parameters used in the automated photometric systems were: temperature, 37 °C; wavelengths, 340 nm and 415 nm (bichromatic); optical path, 1 cm. The reagents used were: Enzytec™ Acetic acid Cod. E2580 and Enzytec™ Liquid Ethanol Cod. E8340. All analyses were performed in duplicates.

3.2.5. Laboratory-scale fermentation and distillation of SRF

3.2.5.1. Wort production, fermentation and distillation from honey by-products

The three strains showing the best technological performances (low production of H_2S and acetic acid, resistance to high ethanol and KMBS concentrations, ability to grow at low temperature, FR, FV, FP and highest score of sensory overall impression) were evaluated for their ability to ferment mead wort obtained from honey by-products.

The wort used to perform the fermentation trials was produced at the honey company G. Pagliaro Az. Agricola (located in Sortino, Italy) following the traditional process of honey and beeswax manufacturing (Gaglio et al., 2017). The process starts from a mixture of honey by-products (honeycombs and capping wax) and water that are transferred into a steel vat and heated at 85-90 °C for 45 min. This phase is preliminary to the extraction of the watery decoction to be subsequently fermented. To this purpose, aliquots of 24 L of wort were transferred into 25 L sterile batches and transported at fridge temperature to the Agricultural Microbiology laboratory of SAAF Department where wort was stabilized at 4 °C for 12 h. Potassium metabisulphite (HTS Enologia, Marsala, Italy)

was added (50 mg/L) to all treatments except uninoculated treatment, to prevent lactic acid bacteria growth, as described by Roldán et al. (2011). Each selected strain was inoculated individually to achieve a final concentration of 10^7 CFU/mL into wort. A positive control (QA23) was carried out with the starter strain *S. cerevisiae* Lalvin QA23 (Lallemand Inc., Montreal, Canada), which is widely used for mead must fermentation (Schwarz et al., 2020; Pereira et al., 2014; Pereira et al., 2015a). Uninoculated must (UI) represented the negative control, spontaneously fermented in order to simulate the protocol of companies producing SRF. Fermentation temperature was 15±1 °C. All fermentations were carried out in duplicates. Sample collection included wort before and after strain inoculation, after 3, 6, 12, 24 and 48 d of AF, and after distillation. Alcoholic fermentation was considered to be completed when the residual sugar value remained unchanged on two consecutive days. All samples were transferred into sterile plastic jars and transported in a portable fridge to the laboratory of Agricultural Microbiology.

For each treatment, aliquots of 10 L of FHP were subjected to static clarification at 4 °C and then distilled. The production of SRF distillate was performed at University of Palermo using official CE distiller (Exacta + Optech Labcenter Spa, San Prospero, Italy).

3.2.5.2. Strain monitoring

Wort samples collected during AF were subjected to microbiological analysis in order to investigate the concentration of total yeast. After decimal dilutions into peptone water solution (Sinacori et al., 2014), samples were inoculated onto Wallerstein Laboratory (WL) nutrient agar (Francesca et al., 2014). All media were purchased from Oxoid. Microbiological counts were performed in triplicates. The colonies developed at the highest dilutions of the cell suspensions, having a colony morphology typical of *Saccharomyces* spp. were subjected to genomic DNA extraction as reported by Gaglio et al. (2017). The 5.8S-ITS PCR analysis was performed to confirm isolates belonged to the *Saccharomyces* genus. Dominance of each inoculated starter strain was verified by comparison of the interdelta profiles (Legras and Karst, 2003) of the isolates with the reference starter strain. PCR products visualized as Settanni et al. (2012).

3.2.5.3. Basic chemical determinations

Wort samples were subjected to measurements of pH as reported by Gaglio et al. (2017); total reducing sugars (glucose and fructose), ethanol, glycerol, acetic acids analysis were detected spectrophotometrically using the enzymatic reactions as previously described in section 2.4.2.

The reagents used for determinations of glucose, fructose, glycerol, ammoniacal nitrogen and alpha-amino nitrogen were: Enzytec™ Liquid D-Glucose / D-Fructose Cod. E8160 and Enzytec™ Fluid

Glycerol Cod. E5360, Enzytec™ Fluid Ammonia Cod. E5390 and Enzytec™ Alpha-amino Nitrogen Cod. E2500.

3.2.5.4. Analysis of volatile organic compounds

Determination of the volatile components was a described by Reddy and Dillon (2015). Ten millilitres of samples were mixed with MS SupraSolv® dichloromethane (10 mL) in a 100 mL conical flask and stirred at room temperature for 30 min. The samples were centrifuged at 3000 RPM for 10 min. The aqueous phase was removed and anhydrous sodium sulphate (1 g) was added before centrifuging at 3000 RPM for 10 min. The dichloromethane layer was removed, dried under N_2 gas to 1 mL and injected (1 µL) in GC-MS.

Gas chromatography (GC) and mass spectrometry (GC-MS)

Gas chromatographic analyses were performed in two different GC-MS machines with two different columns. The first one was an Agilent 7000C GC system, fitted with a fused silica Agilent DB-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness), coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 295˚C. Solvent Delay: 5 min. Helium was the carrier gas (1 mL min^{-1}). The second machine was a Shimadzu QP 2010 plus equipped with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) and with a Supelcowax 10 capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness); ionization voltage 70 eV; transfer line temperature, 280˚C. Helium was the carrier gas (1 mL min^{-1}). For both columns, the temperature was initially kept at 40˚C for 5 min, then gradually increased to 250˚C at 2˚C min^{-1} rate, held for 15 min and finally raised to 270˚C at 10˚C min^{-1}. One µL of samples was injected at 250˚C automatically and in the splitless mode; transfer line temperature, 295˚C.

Data analysis and evaluation

The individual peaks were analysed using the GC MS Solution package, Version 2.72. Identification of compounds was carried out using Adams, NIST 11, Wiley 9 and FFNSC 2 mass spectral database. These identifications were also confirmed by other published mass spectra and linear retention indices. The linear retention indices were calculated using a series of *n*-alkanes (C8-C40). In addition, some compounds were confirmed by comparison of mass spectra and retention times with standard compounds.

3.2.5.5. Sensory analysis of distilled FHP

Quantitative descriptive analyses were carried out by panellists to define sensory profiles.

The experimental distilled FHP were brought to ambient temperature and samples (35 mL) were served monadically at 15 °C in standard ISO type tasting glasses, labelled with random three-digit

codes. Water was provided for rinsing between distillates. All evaluations were undertaken between 10.00 and 12.00 a.m. in individual booths (ISO 8589, 2007). The final scores were obtained as a mean of three evaluations with the respective statistical analysis.
Sixteen judges (7 women and 9 men, ranging from 23 to 46 years old) were recruited from Oenologist Associations: National Organization of Wine Taster (ONAV, Italy), Italian Sommelier Association (AIS, Italy) and University of Palermo. The judges had experience in previous sensory evaluations of alcoholic beverages and all of them undertook preliminary tests to determine their sensory performance on basic tastes and the aromas associated with distilled beverage from honey. The sensory analysis of samples was conducted following the methodology described by Jackson (2016): visual perception (appearance), olfactory sensations (odour and flavour), oral sensations (taste and mouthfeel), and overall quality.
The 16 panellists compared the five experimental FHP during different sessions. They consensually generated sensory descriptive attributes regarding appearance, odour, flavour, taste, and overall quality in several sessions. The panellists were also trained for the identification of off-odours and off-flavour (Issa-Issa et al., 2020; Jackson, 2016). The panellists generated a consensual descriptive ballot for samples in which the descriptors were associated with a 9 cm unstructured scale anchored at the left and right extremes with the terms "none/weak" and "strong", respectively (Biasoto et al., 2014; Jackson, 2016; Stone et al., 2008). The samples were evaluated in distinct tasting sessions carried out on successive days. Overall, each judge evaluated each sample with three repetitions. For each repetition, a different bottle of FHP was opened. The Cochran and Cox (1957) incomplete balanced block design was applied to control the contrast effect amongst the samples.

3.2.6. Statistical and explorative multivariate analysis

ANOVA test was applied to identify significant differences among chemical parameters monitored at the end of AF of FHP, microbiological counts, parameters of honey by-product distillates and sensory attributes. The post-hoc Tukey's method was applied for pairwise comparison. Statistical significance was attributed to $P < 0.05$ (Mazzei et al., 2013).
In addition, an explorative multivariate analysis was conducted to perform a strain differentiation based on *in vitro* technological screening and micro-fermentations tests. An agglomerative hierarchical clustering (AHC) was carried out for grouping the strains according to their dissimilarity, measured by Euclidean distances and Ward's method. The input matrix used for AHC included values of growth in presence of ethanol (12, 14, 16% v/v), ethanol and KMBS resistance (14 and 16 % v/v at 15 and 25 mg/L), production of H_2S and acetic acid, growth at 10 and 15 °C, FR, FV and FP.
In order to graphically represent the concentrations of VOCs, a heat map clustered analysis (HMCA), based on hierarchical dendrogram with heat map plot, was employed to represent the individual

content values contained in the data matrix as colours. The heat map was generated using ascendant hierarchical clustering based on Ward's method and Euclidian distance at 0.25 interquartile range to show the similarities between VOCs and distillates obtained from different FHP treatments. The relative VOC concentrations were depicted by colour intensity from yellow (lowest concentration) to red (highest concentration).

The XLStat software version 2020.3.1 (Addinsoft, New York, USA) was used for statistical data processing and graphic constructions.

3.3. RESULTS AND DISCUSSION

3.3.1. Typing of *S. cerevisiae* strains

The 612 isolates belonging to the species *S. cerevisiae* and previously identified from SRF (Gaglio et al., 2017) were subjected to intraspecific characterization. The interdelta technique as a method of typing *S. cerevisiae* strains isolated from honey by-products and spontaneous fermentations of decoction allowed the differentiation of all 612 isolates obtained from the different samples. This technique is frequently applied for typing *S. cerevisiae* strains associated to fermented beverages (Tristezza et al., 2014; Ruiz-Terán et al., 2019). The interdelta analysis was able to separate the isolates into 91 groups, while microsatellite multiplex PCR recognized only 61 different clusters. Fig. 1 shows the dendrogram of 91 interdelta profiles, which were considered different strains. All strains were differentiated into nine main clusters but the majority of them grouped into four mega groups (II, IV, V and IX). Strains of clusters IX and I showed the highest levels of dissimilarity.

3.3.2. In vitro technological selection of *S. cerevisiae* strains

Studies describing the selection of starter yeasts used for mead production are relatively limited. In the last decade, only Pereira et al. (2009) and Schwarz et al. (2020) have focused on this aspect.

The 91 *S. cerevisiae* strains were screened for their fermentative characters. The majority of strains (n=62) showed a very low production of H_2S on Biggy agar plates (white-beige brown colony). All strains showed intense growth at low temperature (10 and 15 °C), in presence of 14% (v/v) ethanol and high concentration (15-25 mg/L) of KMBS. Moreover, 39 strains grew in the presence of 16% (v/v) ethanol; 34 and 17 of which were also able to grow in the presence of 15 and 25 mg/L of KMBS, respectively. The majority of strains showed a low production of acetic acid. This characteristic is very important, since a low concentration of acetic acid is desirable for the production of quality mead. However, low YAN values in the honey-must may lead to higher volatile acidity (Roldán et al., 2011).

In order to select a group of strains to be tested in mead micro-fermentation, the results from the technological screening were further investigated by multivariate statistical analysis (Fig. 2). Based on the dendrogram from AHC analysis, all strains were grouped into three main clusters (Fig. 2a) with a total absolute variance of 0.671. Each cluster was represented by the strains possessing similar *in vitro* technological characteristics. Cluster 3 included 14 strains (SPF14, SPF16, SPF17, SPF21, SPF40, SPF41, SPF42, SPF50, SPF51, SPF52, SPF158, SPF159, SPF162 and SPF163) characterized by resistance to 16% (v/v) ethanol, cross resistance at 16% (v/v) ethanol with 25 mg/L of KMBS and medium production of H_2S.

The technological parameters calculated during the micro-fermentations allowed to evaluate the fermentation performance of the 14 strains. Through AHC analysis, FR, FV and FP values were used to select potential starter strains suitable for SRF laboratory-scale production. The results (Fig. 2b) divided the 14 strains into 3 clusters. Cluster 3, composed by the strains SPF21, SPF42, and SPF 159, differed from clusters 1 and 2 for the higher FR and PV at 10 and 15 °C. Accordingly, the strains SPF21, SPF42, and SPF159 were selected as starter strains to ferment the wort obtained after boiling honey by-products following the protocol for SRF production. The three strains (SPF21, SPF42 and SPF159) showed high fermentative performance for mead production (3.3 % of total strains). Similar proportions were obtained by Caridi et al. (1999) who found only four strains out of 122 *Saccharomyces* spp. strains (3.3 % of total strains) suitable to be used as starters for mead production.

Fig. 1. Cluster analysis of the profiles obtained by PCR inter-delta region from 91 *Saccharomyces cerevisiae* strains isolated from fermented honey by-products.

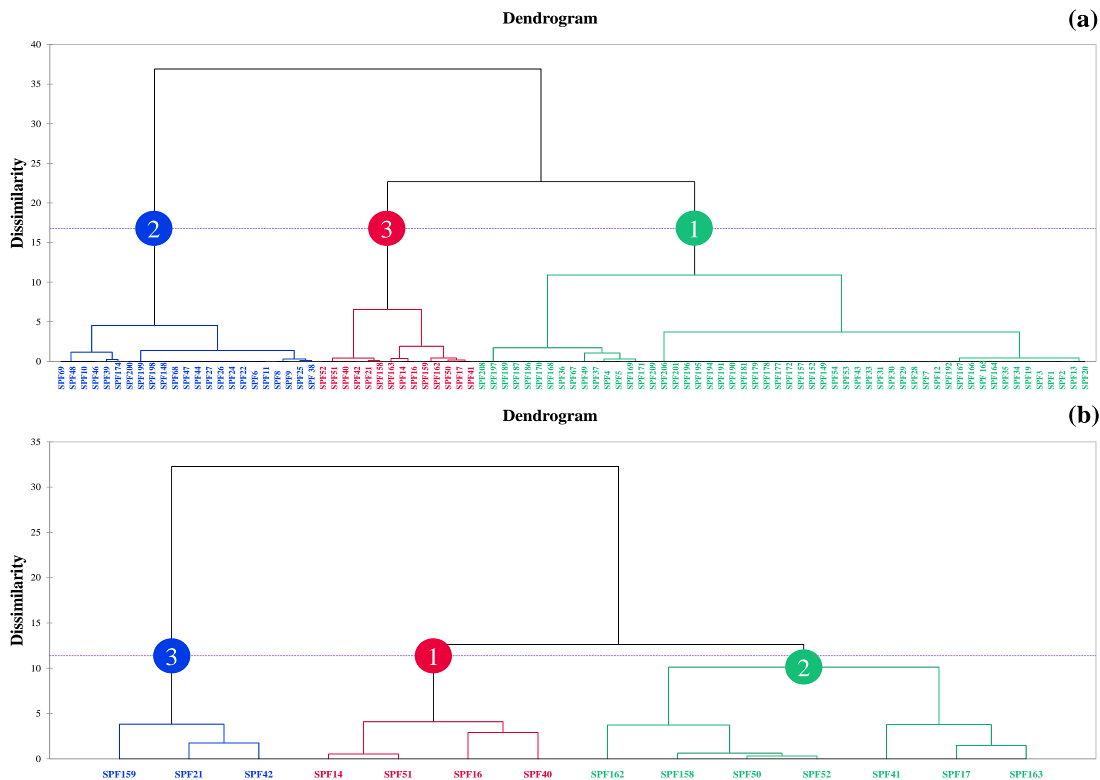


Fig. 2. Dendrogram *of in vitro* technological screening of *S. cerevisiae* strains: **(a)** dendrogram that groups the different strains into 3 clusters (1, 2, 3) in relation to the growth in presence of ethanol (12, 14, 16% v/v), ethanol and KMBS resistance (14 and 16 % v/v at 15 and 25 mg/L), production of H_2S and acetic acid, growth at 10 and 15 °C; **(b)** grouping of strains after *in vitro* micro-fermentation tests in relation to the detected fermentation parameters (fermentation rate, fermentation vigour and fermentation purity).

3.3.3. Laboratory-scale production and distillation of FHP

The decoction composition before strain inoculation was characterized by pH 3.39, 206.83 g/L RS content, 79.03 g/L ammonium nitrogen and 20.11 g/L alpha-amino nitrogen. The decoction showed double or triple yeast assimilable nitrogen (YAN) content than that commonly found in some honey-musts reported in the literature (Mendes-Ferreira et al., 2010; Sottil et al., 2019). Nitrogen is the most important nutrient for yeast growth and has a significant impact on AF. The amount of YAN influences both fermentation kinetics and the organoleptic profile of mead (Mendes-Ferreira et al., 2010). The minimum amount of YAN to ensure a regular fermentation of honey-musts is 250 mg/L (Maugenet, 1964); below these levels the process suffers of nitrogen deficiency limiting the growth of yeast and the fermentation rate (Pereira et al., 2015b).

In all treatments (Fig. 3), immediately after inoculation, the levels of presumptive *Saccharomyces* spp. ranged between 7.2 and 7.4 Log CFU/mL, while, in UI the cell count was below the detection limit. During AF of the inoculated treatments, yeast populations were in the range 7 – 8 Log, with a slight decrease observed at the final stages of AF. In the UI treatment, yeasts were detected after 6 d of AF (2.1 Log CFU/mL), reaching a maximum concentration of 5.7 Log CFU/mL at day 48. Although the composition of the matrix to be fermented is different from that of the honey-must, the

growth dynamics of the presumptive *Saccharomyces* spp. populations were similar to those observed during AF by other authors (Almeida et al., 2020).

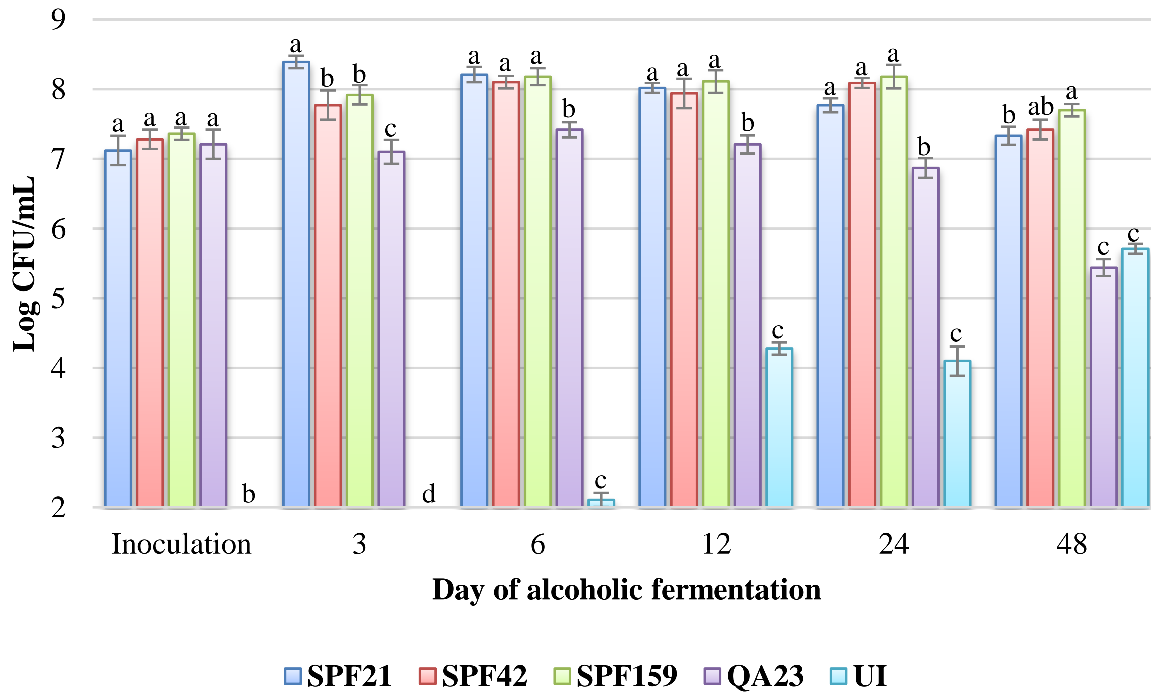


Fig. 3. Microbiological counts (Log CFU/mL) of FHP treatments fermented with *S. cerevisiae* (SPF21, SPF42, SPF159 and QA23) and uninoculated treatment (UI).
Results indicate mean values ± SD of three determinations.
At the same fermentation time, different letters indicate statistically significant values determined with Tukey's test ($P < 0.05$).

To evaluate the persistence of the inoculated yeast strains, interdelta profiles of the isolates from plate counts were compared to those of the pure yeast cultures. The direct comparison of the band patterns of the isolates from all treatments performed with single strain inoculation (SPF21, SPF42, SPF159 and QA23) indicated that the strain inoculated dominated AF. None of the four interdelta profiles from the inoculated yeast strains were found to be associated to any isolate from the control UI, confirming that the dominant microbiota of this treatment did not include the strains used to drive experimental trials AF.

Results of chemical analysis at the end of AF are reported in Table 1. No statistically significant difference was found for pH values. The highest values in acetic acid were observed in the UI treatment (2.87 g/L), whereas the inoculated treatments showed values in the range of 0.28-0.72 g/L. The highest acetic acid concentration observed amongst the inoculated treatments was 0.72 g/L in QA23. Probably, the oenological origin of the strain might cause the increase in volatile acidity (Roldán et al., 2011). The acetic acid values observed in the treatments inoculated with the other strains (SPF21, SPF42 and SPF159) were lower than those measured during the spontaneous AF conducted by Gaglio et al. (2017) but also lower than in the UI treatments of this experiment.

The treatment inoculated with SPF21 showed the lowest values of RS (21.10 g/L) and the highest amounts of ethanol (12.08 % v/v) and glycerol (10.15 g/L).

After static clarification, the volume subjected to distillation varied significantly among the treatments from 9.11 L (QA23) to 9.47 L (SPF21). After distillation, the distilled FHP content was in the range of 0.54-1.02 L. The highest ABV content was obtained in treatments SPF21 (89.00 % v/v) and SPF42 (88.00 % v/v), while the other treatments showed lower values and ranged from 83-85 % v/v. Consequently, the highest Y value was observed in SPF21 (10.75%) and the lowest in UI (5.90%).

Table 1. Chemical parameters monitored at the end of alcoholic fermentation (48 days) of honey by-product decoction and after distillation.

Parameters	Treatments					Statistical significance
	SPF21	SPF42	SPF159	QA23	UI	
FHP						
pH	3.43±0.03^{a}	3.41±0.11^{a}	3.42±0.10^{a}	3.51±0.11^{a}	3.43±0.13^{a}	N.S.
AA	0.28±0.11^{c}	0.37±0.10^{c}	0.39±0.10^{c}	0.72±0.13^{b}	2.87±0.10^{a}	***
RS	21.10±0.35^{e}	38.21±1.01^{c}	32.31±1.17^{d}	78.21±1.41^{b}	97.11±0.98^{a}	***
ET	12.08±0.28^{a}	10.87±0.36^{b}	11.47±0.27^{b}	8.38±0.29^{c}	6.82±0.14^{d}	***
GL	10.15±0.31^{a}	8.41±0.37^{b}	8.97±0.39^{b}	6.74±0.28^{c}	8.32±0.41^{b}	***
Distillate						
FHP	9.47±0.03^{a}	9.12±0.07^{b}	9.43±0.09^{a}	9.11±0.02^{b}	9.22±0.05^{b}	*
D-FHP	1.02±0.03^{a}	0.86±0.02^{c}	0.96±0.04^{b}	0.66±0.03^{d}	0.54±0.03^{e}	***
ABV	89.00±0.80^{a}	88.00±0.75^{a}	85.00±1.20^{b}	83.00±1.10^{b}	84.00±1.10^{b}	***
Y	10.75±0.04^{a}	9.48±0.03^{c}	10.17±0.04^{b}	7.29±0.02^{d}	5.90±0.04^{e}	***

Result indicates mean value ± standard deviation.
Data within a line followed by the same letter are not significantly different according to Tukey's test.
P value: *, P < 0.05; ***, P < 0.001; N.S., not significant.
Abbreviations: AA, acetic acid (g/L); ABV, Alcohol by Volume (% v/v); D-FHP, distilled FHP (L); ET, ethanol (% v/v); FHP, fermented honey by-products (L); GL, glycerol (g/L); RS, reducing sugars (g/L); Y, yield (%).
Codes: SPF21, SPF42 and SPF159 refer to codes of selected strains inoculated into experimental treatments; QA23 refers to commercial strain as positive control treatments; UI, uninoculated treatment subjected to spontaneous fermentation.

3.3.4. VOCs composition of distilled FHP

VOCs profiles of the SRF obtained with strains SPF21, SPF42, SPF159, QA23 and UI are reported in Table 2.

Alcohols represent the quantitatively most abundant class of VOCs in all samples and the most complex VOC group with eleven different compounds identified. The lowest alcohol percentage (49.52%) was observed for the sample spontaneously fermented. In the inoculated samples the quantity of alcohols varied from 82.68% in SPF159 to 98.35% in SPF42, comparable to that present in the control strain. 3-methyl-1-butanol is the alcohol highest in abundance in SPF21 (75.12%) and in QA23 (70.26%) treatments while only a 5.47% was observed for the SPF159 treatment. In the latter sample, the major alcohol present was 2-methyl-1-butanol (38.51%), absent in the other treatments, together with 2-methyl-1-propanol (10.61%) and phenylethyl alcohol (23.06%), present

in lower quantity in the other ones. 3-methyl-1-butanol (31.12%) is the second major alcohol present in SPF42, that is the only sample containing the higher alcohol hexadecanol (32.9%) and octadecanol (24.75%). The presence of 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol was also observed in honey spirit (Anjos et al., 2020).
Carbonyl compounds represented the second most abundant group of VOCs (6.73% in SPF21, 0.60% in SPF42, 5.10% in SPF159, respectively) which were lower than in QA23 (7.69%) and UI (8.41%), consisting mainly of high aldehydes (hexadecanal, heptadecanal and octadecanal). Hexadecanal and octadecanal were identified in honey and wax samples respectively (Bentivenga et al., 2004).

Table 2. Concentration of VOCs derived from distilled FHP

KRI[a]	KRI[b]	Ident.[c]	Compounds[d]	Treatments				
				SPF21	SPF42	SPF159	QA23	UI
			Σ Alcohols	**89.50**	**98.35**	**82.68**	**86.52**	**49.52**
1060	602	1.2	2-methyl-3-buten-2-ol	2.55	1.28	4.66	2.15	3.90
1106	619	1.2	2-methyl-1-propanol	8.42	3.92	10.61	5.78	7.08
1136	658	1.2	1-butanol	0.20	0.11	0.30	0.30	n.d.
1186	729	1.2	3-methyl-1-butanol	75.12	31.12	5.47	70.26	23.80
1200	730	1.2	2-methyl-1-butanol	n.d.	n.d.	38.51	n.d.	n.d.
1221	735	1.2	3-methyl-3-buten-1-ol	n.d.	n.d.	n.d.	0.14	0.20
1309	732	1.2	3-methyl-2-buten-1-ol	n.d.	n.d.	n.d.	0.19	n.d.
1846	1027	1.2	Benzyl alcohol	n.d.	n.d.	0.07	n.d.	n.d.
1871	1097	1.2	Phenylethyl alcohol	3.21	4.27	23.06	7.70	14.54
2329	1875	1.2	Hexadecanol	n.d.	32.90	n.d.	n.d.	n.d.
2540	2076	1.2	Octadecanol	n.d.	24.75	n.d.	n.d.	n.d.
			Σ Phenols	**0.73**	**0.16**	**2.95**	**0.80**	**0.55**
2289	1512	1.2	2,4-di-tert-butylphenol	0.73	0.16	2.95	0.80	0.55
			Σ Ethers	**1.13**	**n.d.**	**4.31**	**3.14**	**n.d.**
2025	1369	1.2	3,4-dimethoxystyrene	1.04	n.d.	3.50	3.14	n.d.
2217	1311	1.2	4-hydroxy-3-methoxystyrene	0.09	n.d.	0.81	n.d.	n.d.
			Σ Aldehydes	**6.73**	**0.60**	**5.10**	**7.69**	**6.71**
2129	1803	1.2	Hexadecanal	2.20	0.60	1.70	1.97	2.74
2235	1917	1.2	Heptadecanal	0.10	n.d.	n.d.	n.d.	n.d.
2341	2017	1.2	Octadecanal	4.43	n.d.	3.40	5.72	3.97
			Σ Ketones	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**1.70**
1248	715	1.2	3-hydroxy-2-butanone	n.d.	n.d.	n.d.	n.d.	1.70
2312	1376	1.2	1,3-diacetylbenzene	n.d.	n.d.	n.d.	n.d.	n.d.
			Σ Esters	**0.41**	**n.d.**	**0.92**	**0.22**	**0.21**
1118	869	1.2	3-methyl-1-butyl acetate	0.03	n.d.	n.d.	n.d.	n.d.
1779	1258	1.2	Phenethyl acetate	0.36	n.d.	0.92	0.22	0.21
1784	1199	1.2	Methyl salicylate	0.02	n.d.	n.d.	n.d.	n.d.
			Σ Carboxylic Acids	**n.d.**	**n.d.**	**0.28**	**n.d.**	**39.93**
1406	-	1.2	Acetic acid	n.d.	n.d.	0.28	n.d.	37.73
2680	1768	1.2	Tetradecanoic acid	n.d.	n.d.	n.d.	n.d.	2.20
			Σ Nitriles	**1.40**	**0.40**	**1.14**	**1.47**	**1.04**
2150	1683	1.2	Tetradecanenitrile	0.12	n.d.	n.d.	n.d.	n.d.
2358	1890	1.2	Hexadecanenitrile	0.45	n.d.	0.57	0.46	0.32
2523	2071	1.2	Octadecanenitrile	0.83	0.40	0.57	1.01	0.72
			Σ Others	**n.d.**	**n.d.**	**2.21**	**n.d.**	**n.d.**
1440	1254	1.2	1,3-di-tert-butylbenzene	n.d.	n.d.	2.21	n.d.	n.d.
			Total compounds	**99.90**	**99.51**	**99.59**	**99.84**	**99.66**

[a]KRI: Supercowax10 column; [b]KRI: DB5-MS column; [c] Ident.: 1= retention index identical to bibliography; 2= identification based on comparison of MS; [d]Results indicate mean percentage values of three measurements and are expressed as relative peak areas (peak area of each compound/total area of the significant and common peaks to all samples) × l00.

Abbreviations: n.d., not detected. Codes: SPF21, SPF42 and SPF159 refer to codes of selected strains inoculated into experimental treatments; QA23 refers to commercial strain as positive control treatment; UI, uninoculated treatment subjected to spontaneous fermentation.

The carboxylic acids constituted the second most abundant VOC class in the uninoculated wort, represented almost exclusively by acetic acid. This class was present only in the other samples at concentrations below their respective detection thresholds. The total volatile ester content in these samples was low. They were totally absent in SPF42. 3-methylbutyl acetate (isoamyl acetate) was detected only in sample SPF21. The major ester present is phenethyl acetate.

In the study by Gaglio et al. (2017), the highest concentrations of volatile compounds were found in alcohols, aldehydes and esters. In the present study, the low ester content observed is probably due to the use of selected yeast strains, but this aspect requires further investigation. Amyl alcohols and phenylethyl alcohol were the most abundant alcohols. The different distribution between short-chain and long-chain alcohols such as the aldehydes most likely depends on the use of a different analytical techniques.

Also, nitriles were present at low concentrations (0.40-1.47%). Nitriles are reported to be present in honeys from rape (2-methylpropanenitrile), thyme (phenylacetonitrile), rosemary (3-methylbutanenitrile and phenylacetonitrile), loquat (several short chain nitriles) and in Greek cotton honey (neryl and geranyl nitriles) (Jerković et al., 2015).

Fig. 4 shows the dendrogram from cluster analysis and the heat map based on peak areas.

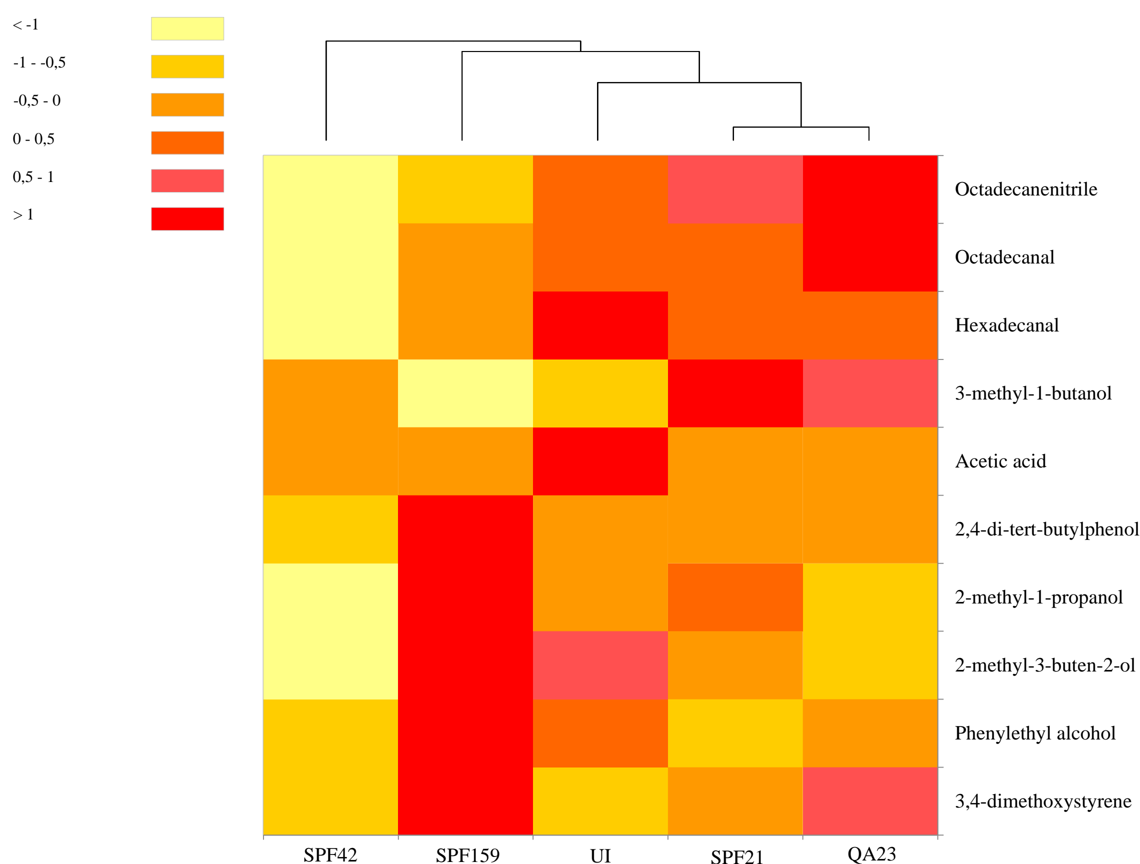


Fig. 4. Distribution of volatile organic compounds (VOCs) among distilled FHP. The hierarchical dendrogram is based on the values of VOCs. The heat map plot depicts the relative percentage of each VOCs (variables clustering on the Y-axis) within each sample (X-axis clustering). Codes: SPF21, SPF42 and SPF159, refers to selected strains inoculated in each experimental treatment; QA23, positive control; UI, uninoculated treatments.

All VOCs with an interquartile range less than 0.25, with low variability, were eliminated to improve the readability of the heat map. Cluster analysis determined the formation of four groups. Among the treatments SPF21 and QA23 a very low level of dissimilarity was found, since they clustered together

into one main group. The other treatments (SFP42, SPF159 and UI) resulted in separate single clusters.

3.3.5. Sensory analysis of distilled FHP

The absence of information regarding quantitative descriptive sensory analysis for SRF distillate led us to use the list of sensory attributes of different distilled beverages (pear brandy, grappa, white rum, honey spirit, vodka, gin, and white tequila) as reported by Donnell et al. (2001), Anjos et al. (2017) and Da Porto (2020).

Results from sensory analysis of distilled FHP are reported in Table 3.

All experimental distilled FHPs showed differences mainly related to the starter strain used. In terms of appearance, the differences were variable in relation to the treatment and in the range of 8.01-8.41 for clarity, whereas colour tonality had values between 2.22-2.53. The results of the appearance attributes therefore revealed the absence of colour tones, which is very common in distillates that are not aged (Anjos et al., 2017).

Treatments SPF21 and SPF42 showed the highest scores for 12 (odour: intensity, persistency, floral, dried flowers, fruity, bitter almond, anise and honey; flavour: intensity and persistency; overall quality: odour; finish: after-smell) and 15 attributes (odour: cantaloupe, fresh vegetable, cloves, caramel and jasmine; taste: sweet; mouthfeel: warmness; flavour: intensity and persistency; overall quality: odour, taste, mouthfeel and flavour; finish: after-smell and after-taste), respectively. Olfactory attributes such as floral, fruity, honey, caramel, jasmine are characteristic of honeys (Anupama et al., 2003). For this reason, there is a direct correlation among the odours generated by the by-products of honey processing and the sensory characteristics of the resulting distillates.

In the UI treatment, odours of carob fruit and walnut husk, absent in treatments inoculated with the different starter yeasts, were perceived. In some cases, the distillates produced odours recognized only in one treatment: cantaloupe and jasmine in treatment SPF42. Treatments inoculated with SPF21, SPF42 and SPF159 did not show the presence of off-odours, while the QA23 treatment (positive control) displayed the presence of the pharmaceutical attributes. Off-odours of burnt caramel (6.10), hydrocarbon (3.72) and sweat (4.21) were detected in UI treatment. The treatments conducted with the selected *S. cerevisiae* strains resulted in a product without off-odours (Mendes-Ferreira et al., 2010) and with a relevant number of attributes rated with high scores by the panellists, which improved the traditional spontaneously fermented distillate.

Table 3. Evaluation of the sensory attributes of the distilled FHP.

Attributes	Treatments					SEM	Statistical significance	
	SPF21	SPF42	SPF159	QA23	UI		Judges	Treatments
Appearance								
Clarity	8.12^{bc}	8.22^{b}	8.41^{a}	8.11^{bc}	8.01^{c}	0.02	***	***
Colour tonality	2.36^{c}	2.46^{b}	2.53^{a}	2.41^{c}	2.22^{d}	0.01	***	***
Odour								
Intensity	8.31^{a}	6.98^{c}	7.61^{b}	6.28^{d}	6.72^{cd}	0.09	***	***
Persistency	8.62^{a}	6.82^{bc}	7.59^{b}	6.20^{cd}	5.37^{d}	0.14	***	***
Floral	5.41^{a}	3.10^{c}	4.01^{b}	2.11^{d}	1.25^{e}	0.18	***	***
Dried flowers	6.51^{a}	2.50^{c}	4.61^{b}	1.87^{c}	1.52^{c}	0.24	***	***
Fruity	7.51^{a}	3.21^{b}	7.10^{a}	7.71^{a}	1.00^{c}	0.34	***	***
Bitter almond	6.81^{a}	4.21^{b}	3.01^{c}	1·00^{d}	2.10^{c}	0.25	***	***
Carob fruit	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	5.51^{a}	0.23	***	***
Cantaloupe	1.00^{b}	6.81^{a}	1.00^{b}	1.00^{b}	1.00^{b}	0.29	***	***
Fresh vegetable	5.21^{bc}	6.81^{a}	5.50^{b}	7.21^{a}	4.37^{c}	0.13	***	***
Walnut husk	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	4.12^{a}	0.16	***	***
Spicy	6.12^{b}	7.01^{ab}	3.31^{c}	6.17^{b}	7.64^{a}	0.19	***	***
Anise	6.78^{a}	1.00^{c}	5.87^{a}	2.88^{b}	1.00^{c}	0.30	***	***
Cloves	3.51^{b}	4.71^{a}	3.10^{b}	3.57^{b}	1.00^{c}	0.15	***	***
Caramel	6.31^{ab}	7.14^{a}	5.51^{b}	3.81^{c}	3.51^{c}	0.18	***	***
Honey	7.10^{a}	5.55^{b}	7.02^{ab}	6.32^{ab}	2.14^{c}	0.23	***	***
Smoky	1.00^{c}	7.64^{a}	1.00^{c}	6.61^{ab}	5.51^{b}	0.35	***	***
Jasmine	1.00^{b}	6.12^{a}	1.00^{b}	1.00^{b}	1.00^{b}	0.26	***	***
Off-odours								
Burnt caramel	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	6.10^{a}	0.25	***	***
Hydrocarbon	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	3.72^{a}	0.14	***	***
Pharmaceutical	1.00^{b}	1.00^{b}	1.00^{b}	3.82^{a}	1.00^{b}	0.14	***	***
Sweat	1.00^{b}	1.00^{b}	1.00^{b}	1.00^{b}	4.21^{a}	0.16	***	***
Taste								
Sweet	5.51^{b}	6.01^{a}	5.78^{ab}	5.97^{a}	4.99^{c}	0.05	***	***
Bitter	2.28^{bc}	2.10^{c}	2.50^{b}	2.15^{c}	3.78^{a}	0.08	***	***
Mouthfeel								
Warmness	7.78^{b}	8.12^{a}	7.89^{b}	7.77^{b}	6.15^{c}	0.09	***	***
Body	7.45^{b}	7.88^{a}	7.82^{a}	7.01^{c}	6.87^{d}	0.05	***	***
Flavour								
Intensity	7.50^{a}	6.80^{a}	7.26^{a}	6.14^{b}	5.17^{c}	0.11	***	***
Persistency	7.82^{a}	8.00^{a}	7.44^{a}	6.68^{b}	5.71^{c}	0.11	***	***
Overall quality								
Odour	7.68^{a}	8.40^{a}	7.03^{a}	5.15^{b}	3.18^{c}	0.24	***	***
Taste	7.15^{ab}	7.98^{a}	7.52^{ab}	6.65^{b}	4.19^{c}	0.17	***	***
Mouthfeel	7.10^{b}	7.55^{a}	7.60^{a}	6.89^{b}	6.15^{c}	0.07	***	***
Flavour	7.48^{ab}	8.22^{a}	7.80^{ab}	6.32^{b}	3.17^{c}	0.23	***	***
Finish								
After-smell	8.01^{a}	7.77^{a}	7.12^{a}	6.21^{b}	4.87^{c}	0.14	***	***
After-taste	7.11^{ab}	8.10^{a}	6.80^{b}	6.12^{b}	3.10^{c}	0.21	***	***

Results indicate mean value.
Data within a line followed by the same letter are not significantly different according to Tukey's test.
P value: ***, $P < 0.001$; **, $P < 0.01$; * $P < 0.05$.
Abbreviations: SEM, Standard error of the mean.
Codes: SPF21, SPF42 and SPF159 refer to codes of selected strains inoculated into experimental treatments; QA23 refers to commercial strain as positive control treatment; UI, uninoculated treatment subjected to spontaneous fermentation.

The attributes describing the taste of the distillates differed between the treatments. High values were observed in SPF21, SPF42 and QA23 for sweet. Mouthfeel also showed statistically significant differences for almost all treatments. The attribute warmness showed the highest value (8.42) in

SPF42 treatment, while body (7.88 and 7.82) in SPF42 and SPF159, respectively. Flavour values for the attributes intensity and persistence were evaluated with higher scores in treatments inoculated with the SPF strains. This trend was also detected for the attributes that describe the overall quality and finish.

3.4. CONCLUSIONS

The unfavourable conditions during "Spiritu re fascitrari" production cause stress to the indigenous decoction yeasts, which leads to the development of unpleasant odours and flavours. Laboratory-scale productions performed in this study clearly showed the suitability of three *S. cerevisiae* strains (SPF21, SPF42, and SPF159) to ferment honey by-product decoction, generating a product with no detectable sensory defects. Each strain gave rise to a unique VOC profile, which made it possible to diversify the experimental productions. In conclusion, the isolation of *S. cerevisiae* strains from honey wastes allowed to overcome the limitations to produce a high quality SRF product.

3.5. REFERENCES

Almeida, E.L.M.D., Moreira E Silva, G., Vassalli, I.D.A., Silva, M.S., Santana, W.C., Silva, P.H.A.D., Eller, M.R., 2020. Effects of nitrogen supplementation on *Saccharomyces cerevisiae* JP14 fermentation for mead production. Food Sci. Technol. 40, 336-343.

American Mead Makers Association, 2017. What's the Buzz? 2017 mead Industry Report. Mead-Makers.org.

Anjos, O., Frazão, D., Caldeira, I., 2017. Physicochemical and sensorial characterization of honey spirits. Foods, *6*(8), 58.

Anjos, O., Santos, R., Estevinho, L.M., Caldeira, I., 2020. FT-RAMAN methodology for the monitoring of honeys' spirit distillation process. Food Chem. 305, 125511.

Anupama, D., Bhat, K.K., Sapna, V.K., 2003. Sensory and physico-chemical properties of commercial samples of honey. Int. Food Res. J. 36(2), 183-191. https://doi.org/10.1016/S0963-9969(02)00135-7.

Araújo, T.M., Souza, M.T., Diniz, R.H.S., Yamakawa, C.K., Soares, L.B., Lenczak, J.L., Oliveira, J.V.D.C., Goldman, G.H., Barbosa, E.A., Campos, A.C.S., Brandão R.L., Castro, I.M., 2018. Cachaça yeast strains: alternative starters to produce beer and bioethanol. Antonie Van Leeuwenhoek 111(10), 1749-1766.

Beer Judge Certification Program (BJCP), 2017.

Bentivenga, G., D'Auria, M., Fedeli, P., Mauriello, G., Racioppi, R., 2004. SPME-GC-MS analysis of volatile organic compounds in honey from Basilicata. Evidence for the presence of pollutants from anthropogenic activities. Int. J. Food Sci. 39(10), 1079-1086.

Biasoto, A.C.T., Netto, F.M., Marques, E.J.N., da Silva, M.A.A.P., 2014. Acceptability and preference drivers of red wines produced from *Vitis labrusca* and hybrid grapes. Food Res. Int. 62, 456-466.

Caridi, A., Fuda, S., Postorino, S., Russo, M., Sidari, R., 1999. Selection of *Saccharomyces sensu stricto* for mead production. Food Technol. Biotechnol. 37(3), 203-207.

Ciani, M., Maccarelli, F., 1998. Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. World J. Microbiol. Biotechnol. 14(2), 199-203.

Cochran, W.G., Cox, G.M., 1957. Experimental designs (2nd ed.). New York, Wiley.

Czabaj, S., Kawa-Rygielska, J., Kucharska, A.Z., Kliks, J., 2017. Effects of mead wort heat treatment on the mead fermentation process and antioxidant activity. Molecules 22(5), 803.

Da Porto, C., 2002. Volatile composition of 'grappa low wines' using different methods and conditions of storage on an industrial scale. Int. J. Food Sci. Technol. 37(4), 395–402.

Donnell, E.M., Hulin-Bertaud, S., Sheehan, E.M., Delahunty, C.M., 2001. Development and learning process of a sensory vocabulary for the odor evaluation of selected distilled beverages using descriptive analysis. J. Sens. Stud. 16 (4), 425–445.

Francesca, N., Chiurazzi, M., Romano, R., Aponte, M., Settanni, L., Moschetti, G., 2010. Indigenous yeast communities in the environment of "Rovello bianco" grape variety and their use in commercial white wine fermentation. World J. Microbiol. Biotechnol. 26(2), 337-351.

Francesca, N., Sannino, C., Settanni, L., Corona, O., Barone, E., Moschetti, G., 2014. Microbiological and chemical monitoring of Marsala base wine obtained by spontaneous fermentation during large-scale production. Ann. Microbiol. 64(4), 1643-1657.

Gaglio, R., Alfonzo, A., Francesca, N., Corona, O., Di Gerlando, R., Columba, P., Moschetti, G., 2017. Production of the Sicilian distillate "Spiritu re fascitrari" from honey by-products: An interesting source of yeast diversity. Int. J. Food Microbiol. 261, 62-72.

Issa-Issa, H., Guclu, G., Noguera-Artiaga, L., López-Lluch, D., Poveda, R., Kelebek, H., Selli, S., Carbonell-Barrachina, Á.A., 2020. Aroma-active compounds, sensory profile, and phenolic composition of Fondillón. Food Chem. 316, 126353.

ISO 8589 (2007). Sensory analysis - General guidance for the design of test rooms. Geneva. Switzerland: International Organization for Standardization.

Jackson, R.S. 2016. Wine tasting. A professional handbook, third edition. Eds. Cool Climate enology and Viticulture Institute, Brock University, St. Catharines, Ontario, Canada.

Jerković, I., Marijanović, Z., Kranjac, M., Radonić, A., 2015. Comparison of different methodologies for detailed screening of *Taraxacum officinale* honey volatiles. Nat. Prod. Commun. 10(2), 357-360.

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. Appl. Environ. Microbiol. 61(2), 461-467.

Legras, J., Karst, F., 2003. Optimisation of interdelta for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiol. Lett. 221, 249-255.

Maugenet, J., 1964. L'hydromel. Les Annales de l'Abeille, 7(3), 165-179.

Mazzei, P., Spaccini, R., Francesca, N., Moschetti, G., Piccolo, A., 2013. Metabolomic by ^{1}H NMR spectroscopy differentiates "Fiano Di Avellino" white wines obtained with different yeast strains. J. Agric. Food Chem. 61(45), 10816-10822.

Mendes-Ferreira, A., Cosme, F., Barbosa, C., Falco, V., Inês, A., Mendes-Faia, A., 2010. Optimization of honey-must preparation and alcoholic fermentation by *Saccharomyces cerevisiae* for mead production. Int. J. Food Microbiol. 144(1), 193-198.

Moschetti, G., Corona, O., Gaglio, R., Squadrito, M., Parrinello, A., Settanni, L., Barone, E., Francesca, N., 2016. Use of fortified pied de cuve as an innovative method to start spontaneous alcoholic fermentation for red winemaking. Aust. J. Grape Wine Res. 22, 36–45.

Osservatorio Nazionale Miele, 2020. Il valore della terra: agricoltura e nuova ruralità, economia e sostenibilità, qualità e consumo consapevole. Miele andamento produttivo e di mercato per la stagione 2019.

Peepall, C., Nickens, D.G., Vinciguerra, J., Bochman, M.L., 2019. An organoleptic survey of meads made with lactic acid-producing yeasts. Food microbial. 82, 398-408.

Pereira, A. P., Dias, T., Andrade, J., Ramalhosa, E., Estevinho, L. M., 2009. Mead production: Selection and characterization assays of *Saccharomyces* cerevisiae strains. Food Chem. Toxicol. 47(8), 2057-2063.

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2014. Effect of *Saccharomyces cerevisiae* cells immobilisation on mead production. LWT - Food Sci. Technol. 56, 21–30.

Pereira, A.P., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A., 2015a. Improvement of mead fermentation by honey-must supplementation. J. Inst. Brew. 121(3), 405-410.

Pereira, A. P., Mendes-Ferreira, A., Oliveira, J. M., Estevinho, L. M., Mendes-Faia, A., 2015b. Mead production: effect of nitrogen supplementation on growth, fermentation profile and aroma formation by yeasts in mead fermentation. J. Inst. Brew. 121(1), 122-128.

Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T., Estevinho, L.M., 2011. Mead production: tradition versus modernity. Adv. Food Nutr. Res. 63, 101–118.

Reddy, S., Dillon, T., 2015. Profiling of Aroma Components in Wine Using a Novel Hybrid GC/MS/MS System. PerkinElmer, Inc. Waltham, USA.

Roldán, A., Van Muiswinkel, G. C. J., Lasanta, C., Palacios, V., Caro, I., 2011. Influence of pollen addition on mead elaboration: Physicochemical and sensory characteristics. Food Chem. 126(2), 574-582.

Ruiz-Terán, F., Martínez-Zepeda, P.N., Geyer-de la Merced, S.Y., Nolasco-Cancino, H., Santiago-Urbina, J.A., 2019. Mezcal: indigenous *Saccharomyces cerevisiae* strains and their potential as starter cultures. Food Sci. Biotechnol., 28(2), 459-467.

Schwarz, L. V., Marcon, A. R., Delamare, A. P. L., Agostini, F., Moura, S., Echeverrigaray, S., 2020. Selection of low nitrogen demand yeast strains and their impact on the physicochemical and volatile composition of mead. J. Food Sci. Technol. 57(8), 2840-2851.

Settanni, L., Sannino, C., Francesca, N., Guarcello, R., Moschetti, G., 2012. Yeast ecology of vineyards within Marsala wine area (western Sicily) in two consecutive vintages and selection of autochthonous *Saccharomyces cerevisiae* strains. J. Biosci. Bioeng. 114(6), 606-614.

Sinacori, M., Francesca, N., Alfonzo, A., Cruciata, M., Sannino, C., Settanni, L., Moschetti, G., 2014. Cultivable microorganisms associated with honeys of different geographical and botanical origin. Food Microbiol. 38, 284–294.

Sottil, C., Salor-Torregrosa, J. M., Moreno-Garcia, J., Peinado, J., Mauricio, J. C., Moreno, J., Garcia-Martinez, T., 2019. Using *Torulaspora delbrueckii*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus* wine yeasts as starter cultures for fermentation and quality improvement of mead. Eur. Food Res. Technol. 245(12), 2705-2714.

Stone, H., Sidel, J., Oliver, S., Woolsey, A., Singleton, R.C., 2008. Sensory evaluation by quantitative descriptive analysis. Descriptive sensory analysis in practice 28, 23-34.

Tristezza, M., Fantastico, L., Vetrano, C., Bleve, G., Corallo, D., Grieco, F., Mita, G., 2014. Molecular and technological characterization of *Saccharomyces cerevisiae* strains isolated from natural fermentation of Susumaniello grape must in Apulia, Southern Italy. Int. J. Microbiol. 2014, 1-11.

Vaudano, E. Garcia-Moruno, E., 2008. Discrimination of *Saccharomyces cerevisiae* wine strains using microsatellite multiplex PCR and band pattern analysis. Food Microbiol. 25, 56–64.

CHAPTER 4

Use of Saccharomyces and non-Saccharomyces strains isolated from honey by-products to improve and stabilize the quality of mead produced in Sicily

ABSTRACT

Mead is an alcoholic beverage produced by alcoholic fermentation of honey-must. The starter yeasts that are commonly used for the alcoholic fermentation of honey-must are oenological *Saccharomyces cerevisiae* strains. The objective of the present work was, for the first time, to apply yeasts of melliferous origin to improve the taste-olfactory attributes of mead. For this purpose, three experimental productions were set up, which included: (i) single inoculation of *S. cerevisiae*; (ii) single inoculation of *Hanseniaspora uvarum*; (iii) sequential inoculation of *H. uvarum* and *S. cerevisiae*. Two control trials were performed, using a commercial strain of *S. cerevisiae* of oenological origin and a spontaneous fermentation. The results of the chemical parameters showed differences between the trials in terms of residual sugars, acetic acid, glycerol, ethanol and volatile organic compounds. Sensorial analysis also showed a high heterogeneity among trials. The attributes of sweetness, honey and floral were found in mead fermented with H. uvarum, whereas all meads obtained with *S. cerevisiae* were dry, balanced and without off-odours and off-flavours. The results obtained showed that the application of conventional and non-conventional yeast strains of melliferous origin could be a promising approach to improve the quality of meads.

4.1. INTRODUCTION

Among alcoholic beverages, mead is a very ancient and popular product of Eastern Europe (Poland, Slovenia, and Baltic countries). Nowadays, it is widespread among Northern European populations (Parrini, 2021) and commonly consumed in England, Germany and, in particular, in African countries, including Ethiopia and South Africa (De Rubeis, 2016). Despite its popularity in those countries, mead is almost unknown in some others, like Italy, where it is hardly commercialized. Regarding the production aspects, mead is obtained through the alcoholic fermentation (AF) of honey and possesses a great commercial potential (Ramalhosa et al., 2011).

The quality of mead is affected by several factors that depend on honey production conditions (Gupta and Sharma, 2009; Ukpabi, 2006) and, especially, on the fermentation process; to this purpose, the generation of off-flavours by yeasts represents one of the main issues (Ramalhosa et al., 2011). Recently, many studies focused on the improvement of mead production. The most important parameters to be monitored during mead production include pH, ethanol concentration, volatile acidity, titratable acidity, sugar content and organic acid content (Ramalhosa et al., 2011). The presence of yeasts is fundamental to obtain high-quality products; besides optimal fermentative performances necessary to metabolize sugars and generate alcohol, yeasts are important to produce the desired aromatic compounds. However, also honey variety can significantly influence the flavour of final mead (Li and Sun, 2019).

The issues related to the yeast fermentative efficiency are generally due to nutritional deficiencies that cause stress and can be easily counteracted through addition of nitrogen, minerals and vitamins (Gibson, 2011). In general, the yeasts applied as starters for mead production are *Saccharomyces cerevisiae*, but the use of non-*Saccharomyces* strains to improve the aromatic profile of mead deserves attention (Varela, 2016, Qureshi and Tamhane, 1987). Among non-*Saccharomyces* yeasts, *Torulaspora delbrueckii* and *Lachancea thermotolerans* showed interesting results (Li and Sun, 2019; Sottil et al., 2019). *T. delbrueckii* produces low ethanol concentration leaving high residual sugar contents but preserves the primary aroma of honey (Sottil et al., 2019). Meads obtained by fermentation with *L. thermotolerans* have shown high content of ethyl acetate and *n*-decanoic acid (Li and Sun, 2019).
In general, mead is the alcoholic beverage that mostly suffered of research progress and still needs optimization of production process and stabilization of the microbial populations. Trying to provide useful insights to lead the fermentation steps, the aim of this work was to evaluate the effect of *Saccharomyces* and non-*Saccharomyces* strains from honey environments for the production of non-pasteurized Sicilian mead. Specifically, *S. cerevisiae* and *Hanseniaspora uvarum* strains isolated from honey by-products, previously selected, and technologically characterized were used to replace wine- and beer-related starters commonly used to process this kind of alcoholic product. A sequential inoculum of the selected strains was tested. The microbiological evolution and chemical parameters were evaluated during the AF and the sensory aspects and the profiles of volatile organic compounds were measured on the final products.

4.2. MATERIALS AND METHODS

4.2.1. Experimental design

The experimental design of mead production is depicted in Fig. 1.
Briefly, five 8-L volume batches of honey-must, with an original gravity of 18 °KMW and pH of 4.41 was prepared using multifloral honey from "Nettare di Sicilia S.a.s. - Cirrito" company (Caltavuturo, Italy) and bottled water (Levissima, San Pellegrino S.p.A., Valdisotto, Italy). Following the common supplementation of nitrogen sources during mead production (Pereira et al., 2013), 20 g/hL of Nutristart® ORG (Laffort®, Tortona, Italy) and 20 g/hL of Thiazote® PH (Laffort®, Tortona, Italy), were added as supporting nutrients at the beginning of AF and at half of total sugar consumption, respectively.

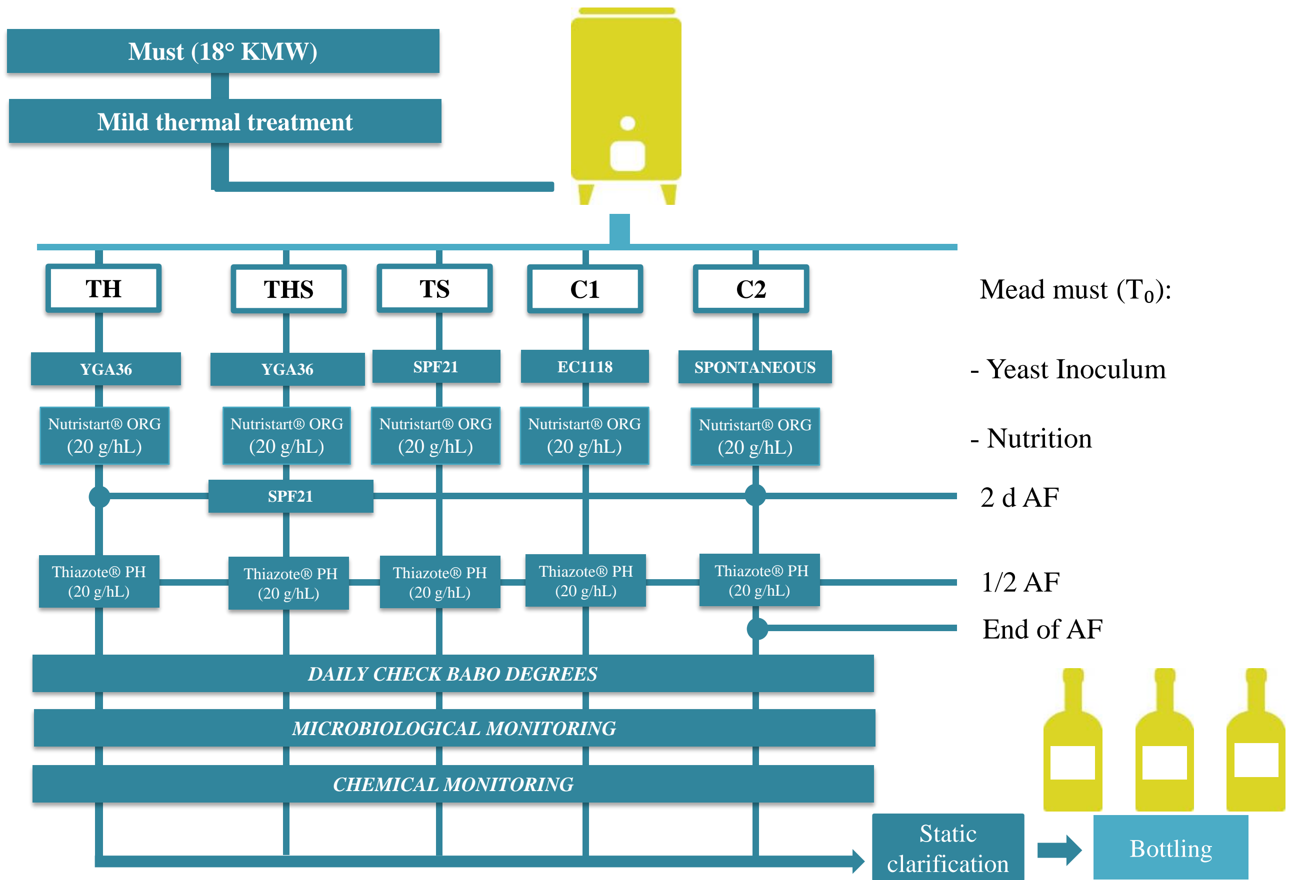


Fig. 1. Experimental plan of mead production.

The three experimental trials (TS, TH and THS) were inoculated as follows: TS, inoculated with monoculture of *S. cerevisiae* SPF21 strain; TH, inoculated with monoculture *H. uvarum* YGA36 strain; THS, sequentially inoculated firstly with YGA36 strain followed, after 48 h, by SPF21 strain. Control C1 trial was inoculated with monoculture of *S. cerevisiae* EC1118 strain. The last batch was left un-inoculated and represented the control trial (C2) for spontaneous fermentation. Prior yeast inoculation, potassium metabisulphite (HTS Enologia, Marsala, Italy) was added (50 mg/L) to all trials except C2, to prevent lactic acid bacteria (LAB) growth (Roldán et al., 2011). The fermentation was carried out at 20 °C under static condition.

S. cerevisiae SPF21 and *H. uvarum* YGA36 yeast strains, both isolated from honey by-product (Gaglio et al., 2017), belongs to the oenological yeast collection of the Department of Agricultural Food and Forest Sciences (SAAF; University of Palermo, Italy). *S. cerevisiae* EC1118 is a commercial strain (Lallemand Inc., Montreal, Canada) for oenological purposes. All strains were reactivated from -80 °C glycerol stocks by plating onto YPD agar. After 3 - 5 d at 25 °C, the strains were pre-cultured overnight in 5 mL YPD and then re-inoculated into sterile flasks containing YPD, where cells were let to grow. The cells were washed twice with Ringer solution to remove trace of

growth broth and inoculated at a final cell density of 2.0×10^6 cells/mL (Holt et al., 2018). Media were purchased from Oxoid (Rodano, Italy).

All experimental fermentation trials were performed in triplicate. Samples were collected at different stages of mead production: honey must before inoculation, honey must after yeast addition, mead during the AF (day 1, 2, 3, 6, 12 and 18), final mead (day 30). All samples were subjected to analysis within 24 h from collection. All analyses were performed in triplicate.

4.2.2. Microbiological analyses and strain typing

The levels of total yeasts were evaluated onto Wallerstein laboratory (WL) nutrient agar, incubated aerobically at 28 °C for 72 h. After growth, all isolates were picked up from the agar plates, purified by successive sub-culturing and subjected to genotypic identification in order to verify the dominance of inoculated strains.

The genetic diversity of *Saccharomyces* isolates was assessed by interdelta analysis (Legras and Karst, 2003), while *H. uvarum* dominance was verified using P80 primer (Capece et al., 2005). All DNA patterns were analysed using the Gel Compare II software (v. 6.1. Applied Maths NV. Sint-Martens-Latem. Belgium) and similarities among patterns were assessed. DNA band profiles showing more than 95% of similarity were considered identical.

4.2.3. Physicochemical analysis

The determination of Babo degree (°KMW) was performed by hydrometer (Polsinelli Enologia Srl, Isola del Liri, Italy). The concentrations of glucose, fructose, ethanol, ammonia nitrogen and alpha-amino nitrogen (TN), glycerol, and acetic acid were determined enzymatically through the analyser iCubio iMagic M9 (Shenzhen iCubio Biomedical Technology Co. Ltd. Shenzhen, China); this system allows automatic reagent mixing, incubation at 37 °C, reading of absorbance at 340 and 415 nm (bichromatic) with 1 cm optical path and determines the concentration of the chemicals by means of a calibration curve. The reagents used were: Enzytec™ Liquid D-Glucose/D-Fructose (Cod. E8160), Enzytec™ Liquid Ethanol (Cod. E8340), Enzytec™ Fluid Glycerol Cod. (E5360), Enzytec™ Acetic acid (Cod. E2580), and Enzytec™ Liquid Ammonia (Cod. E8390). The standard used for the calibrations of apparatus were: Enzytec™ Multi-acid standard automation Cod. E1241 for acetic acid; Enzytec™ Sugar standard Cod. E5450 for glucose and fructose; Enzytec™ Alcohol standard Cod. E5420 for ethanol; Enzytec™ Sugar standard manual Cod. E1242 for glycerol. All reagents and standards were purchased from R-Biopharm AG (Darmstadt. Germany). All samples were diluted until reaching the optimal concentration by direct comparison with the calibration curve.

4.2.4. Determination of volatile organic compounds

The volatile organic compounds were analysed in Solid Phase Micro Extraction (SPME). The Fibre Assembly used was 50/30 µm divinylbenzene (DVB)/carbowax (CAR)/polydimethylsiloxane (PDMS) (Supelco, Bellefonte PA, 19823-0048 USA). Fibre was exposed to 10 mL of sample in 20-mL volume SPME vial (75.5 × 22.5 mm) for 30 min at 40 °C, after 30 min of equilibration time. The desorption time was set from 5 min. Before using, fibre was conditioned and cleaned at 270 °C for 30 min, following instructions from Supelco® (Merk Life Science S.r.l., Milano, Italy). Split-less injection was used. Each measurement was replicated 3 times. The result, expressed in parts per million (ppm), was calculated using a calibration curve established in a range 100 to 0 ppm. The standard curve of ethyl benzoate was $y = 2\times10^9$ with $R^2 = 0.9966$.

Gas chromatographic analyses were performed in Agilent 7000C GC system, fitted with a fused silica Agilent DB-5MS capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness), coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 270 ˚C. Solvent Delay: 0 min. Helium was the carrier gas (1 mL/min).

4.2.5. Sensory analysis

Sensory analysis of the resulting meads was performed by a panel of 10 assessors (4 females and 6 males) aged between 24 and 62 years old, specifically trained for this judging session. Samples were served in glasses, covered to minimize evaporation, following the methodology based on a 9 cm unstructured scale anchored at left and right extremes with the terms "none/weak" and "strong" respectively (Barry et al., 2018; Jackson, 2016). The descriptor used to evaluate the experimental meads were divided into three sections according to Pereira et al. (2019): appearance (colour), aroma (intensity, persistence, fruity, floral, honey, spicy, rancid acetic, oxidized, vinous, and aroma overall impression) and taste (intensity, persistence, sweet, acid, rancid, acetic, oxidized, vinous, and taste overall impression). Colour intensity was visually evaluated by the panellists on a 9 cm scaled anchored at left and right extremes with the terms "straw-yellow" and "amber-yellow". The descriptor "overall quality" was also included for the determination of overall rating, a general evaluation based on the scores of all attributes.

4.2.6. Statistical analysis

ANOVA test was applied to identify significant differences among chemical parameters, microbiological analysis, and sensory evaluation. The post-hoc Tukey's method was applied for pairwise comparison of all data. Statistical significance was attributed to $P < 0.05$ (Mazzei et al., 2010).

An explorative multivariate approach was employed to investigate relationships among data obtained during AF (aroma, taste, colour and chemical parameters) from the different trials.

To graphically represent the concentrations of VOCs, a heat map clustered analysis (HMCA), based on hierarchical dendrogram with heat map plot, was employed to represent the individual content values contained in the data matrix as colours. The heat map was generated using ascendant hierarchical clustering based on Ward's method and Euclidian distance at 0.1 interquartile range to show the similarities between VOCs and meads obtained with the different yeast inoculums. The relative concentrations of VOCs were depicted by colour intensity from yellow (lowest concentration) to red (highest concentration). Heat map analysis of the volatile levels was performed using the autoscaled data (Gaglio et al., 2017).

The agglomerative hierarchical clustering (AHC) of sensory data were performed in order to investigate relationships among trials.

Statistical data processing and graphic construction were performed with the XLStat software version 2020.3.1 (Addinsoft, New York, USA) for excel.

4.3. RESULTS AND DISCUSSION

4.3.1 Monitoring of microbial populations

The evolution of yeast populations during the AF of mead for the different trials is reported in Fig. 2.

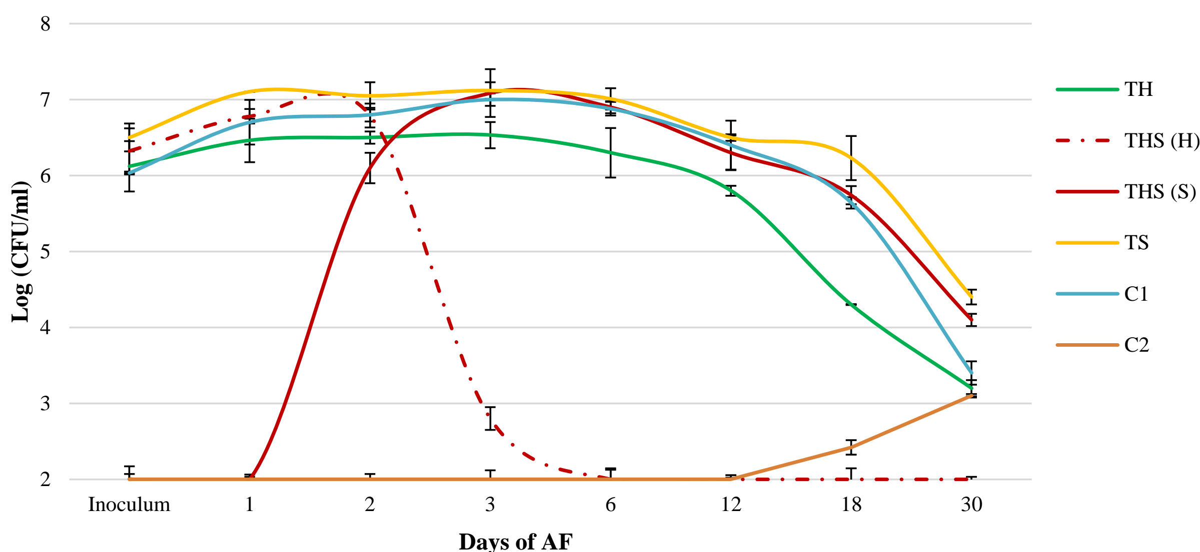


Fig. 2. Monitoring of yeast concentrations during alcoholic fermentation. Mead fermented by: *S. cerevisiae* SPF21 (TS); *H. uvarum* YGA36 (TH); sequential inoculum with YGA36 [THS(H)] and SPF21 [THS(S)]; *S. cerevisiae* EC1118 (C1) and un-inoculated trial subjected to spontaneous fermentation (C2).

The level of total yeasts in honey-must before starter addition was below the detection limit. Plate counts indicated that the initial yeast inoculums were between 6.0 and 6.5 Log CFU/mL. An increase of about 0.5 Log cycles was detected after 24 h for all trials confirming the common yeast dynamics

during honey-must fermentation (Pereira et al., 2015a, b). At the second day of AF, the trial THS was inoculated with *S. cerevisiae* SPF21 at 6.1 Log CFU/mL, as confirmed by plate counts. This addition determined, after further 24 h, a sudden decrease of *Hanseniaspora* spp. population of trial THS until day 6, when these yeasts were undetectable, while *S. cerevisiae* increased up to 7.1 Log CFU/mL. The reduction of *Hanseniaspora* spp. population is due to the nature of the interaction with *S. cerevisiae* (Tristezza et al., 2016), mainly due to competition for nutrients (Domizio et al., 2011), but also to *S. cerevisiae* metabolites that hinder the normal growth of *H. uvarum* (Wang et al., 2015).
S. cerevisiae population levels remained above 6 Log cycles for all trials until day 12. Instead, *H. uvarum* in trial TH showed a remarkable decrease from day 6 onward until day 30 and the AF remained incomplete. At the end of the AF, the highest cell densities were registered for *S. cerevisiae* SPF21 in trial TS (4.4 Log CFU/mL) and THS (4.1 Log CFU/mL), followed by *H. uvarum* YGA36 in trial TH (3.2 Log CFU/mL) and *S. cerevisiae* EC1118 in trial C1 (3.4 Log CFU/mL). Yeast growth dynamics observed in this study was similar to those registered during mead production by Almeida et al. (2020).
The AF was considered completed within 30 days. The values registered were comparable to those reported by Bednarek et al. (2020) who monitored buckwheat honey fermentation by different starter cultures.

4.3.2. Dominance of the starter strains

A total of 1113 colonies from WL agar plates were isolated, purified and classified on the morphological characteristics. Seven hundred and twenty-three colonies shared the morphological characteristics of *Saccharomyces* and, for this reason were referred to as presumptive *Saccharomyces* (PS). 5.8S-ITS amplicon analysis indicated that all isolates were characterized by a fragment of 850 - 880 bp, which is typical of *S. cerevisiae* (Granchi et al., 1999; Fernández-Espinar et al., 2000). The rest of isolates (n = 390) constituted the non-*Saccharomyces* group. Among these, 296 isolates were morphologically considered presumptive *Hanseniaspora* (PH) and were characterized by an ITS amplicon size between 740 and 760 bp, very closed to the 750 bp ITS of *H. uvarum* (Esteve-Zarzoso et al., 1999). All PS and PH isolates were further processed by restriction analysis of 5.8S-ITS region. RFLP profiles of PS isolates obtained by digestion of amplification products with *Cfo*I resulted in two restriction bands (380 and 350 pb). Four bands (320, 230, 180 and 140 bp) were obtained with *Hae*III, while three restriction bands of 365 and 155 bp were obtained when amplicons were digested with *Hinf*I endonuclease. The size of the restriction fragments obtained were highly similar to those reported for *S. cerevisiae* by other authors (Esteve-Zarzoso et al., 1999; Guillamón et al., 1998). Regarding the RFLP profiles of PH isolates obtained after digestion with *Cfo*I, *Hae*III, *Hinf*I and *Dde*I confirmed the results reported by Matraxia et al. (2021) and all cultures were identified as *H. uvarum*.

Interdelta profiles indicated that barely two different *S. cerevisiae* strains were isolated at the highest cell densities from three experimental trials (TS, THS and C1). The direct comparison with the pure cultures indicated that SPF21 and EC1118 strains dominated the fermentation process of the trials corresponding to their inoculation. Following the same strategy, all PH isolates represented a single strain and it was *H. uvarum* YGA36. The frequency of isolation of inoculated strains was particularly high (>95%). All isolates not matching fingerprinting profile of pitched yeasts (< 5%), were isolated from the spontaneously fermented trial (C2).

4.3.3. Physicochemical parameters

The analyses of the physicochemical characteristics of mead from the different trials highlighted several differences in terms of sugar and nitrogen consumption, and glycerol and ethanol production (Table 1). At the 30th day of monitoring, TH and C2 trials showed a residual sugar concentration of 72.62 and 77.80 g/L, respectively, while the other trials were characterized by carbohydrates below 5 g/L. Although sugar consumption kinetics indicated that the strain EC1118 (C1) had the highest consumption rate during the AF, after 30 d, both trials inoculated with SPF21 (TS and THS) reached the same residual sugar concentration. Regarding this carbon source, values below 5 g/L were observed by Li and Sun (2019) during the production of mead with non-*S. cerevisiae* strains and the same *S. cerevisiae* strain (Lalvin EC1118) used in our work.

The highest final ethanol concentration was observed in TS trial with 12.37% (v/v), followed by THS and C1 trials, both with 12.29% (v/v). These ethanol values registered at the end of AF are higher than those recorded in other works on mead fermentation (Li and Sun, 2019; Pereira et al., 2015b).

The value of total nitrogen (TN) measured after the addition of Nutristart® Org was 105.12 mg/L. This parameter decreased rapidly to 1/3 of the initial concentration after 6 d of AF. At this time, TN among trials reached values between 76.4 and 138 mg/L. This difference is due to different nitrogen requirements for different trial, which depends on each yeast metabolic activity. At the end of the process, TN ranged between 19.56 mg/L in trial C1 and 124.06 mg/L in trial TH. In trials which concluded AF, these values ranged between 19.56 and 50.34 mg/L, comparable to the ones (29.17-37.33 mg/L) registered by Pereira et al. (2013) who evaluated different oenological *S. cerevisiae* strains. The final concentration of TN of trial THS was particularly higher than those found in the other trials. The strain SPF21 seemed to necessitate lower nitrogen requirements than EC1118 strain, because this characteristic is strain-dependent (Hernández et al., 2015).

The values of acetic acid measured at the end of the process were variable among trials. The lowest acetic acid concentration was registered in trials C1 (0.29 g/L), TS (0.31 g/L), and THS (0.39 g/L), whereas the highest value was recorded in trial C2 (0.71 g/L). The acetic acid content of the trials C1, TS, and THS was lower than that found by Roldán et al. (2011), who tested the effect of pollen

addition on mead production process, and also below the sensory threshold of 0.7 g/L of acetic acid reported by the same author. Slightly higher values could often be due to low TN concentrations (Vilanova et al., 2007) or to a slowing down of fermentation (Sroka and Tuszyński, 2007). In our case, only the spontaneously fermented trials (C2) showed acetic acid values higher than 0.7 g/L, which represents the sensory threshold limit for wines (Roldán et al., 2011).

Table 1. Chemical parameters monitored during the alcoholic fermentation of mead.

	Treatments					
	TS	**TH**	**THS**	**C1**	**C2**	**S.S.**
D-glucose (g/L)						
Must	74.12 ± 4.60^{a}	74.12 ± 4.60^{a}	74.12 ± 4.60^{a}	74.12 ± 4.60^{a}	74.12 ± 4.60^{a}	n.s.
1d	59.38 ± 5.67^{ab}	67.09 ± 6.88^{ab}	69.20 ± 8.16^{ab}	47.5 ± 4.47^{b}	75.07 ± 12.46^{a}	**
3d	49.46 ± 2.48^{cd}	65.82 ± 3.47^{b}	55.57 ± 5.37^{c}	43.09 ± 1.60^{d}	75.74 ± 7.15^{a}	***
6d	46.20 ± 5.27^{b}	49.04 ± 2.45^{b}	51.38 ± 0.92^{b}	33.25 ± 3.56^{c}	62.17 ± 7.48^{a}	***
12d	18.79 ± 1.89^{bc}	31.91 ± 6.37^{b}	18.80 ± 2.14^{bc}	13.30 ± 1.94^{c}	58.72 ± 6.78^{a}	***
End AF	1.02 ± 0.05^{c}	31.86 ± 3.19^{a}	0.72 ± 0.11^{c}	0.92 ± 0.10^{c}	21.68 ± 3.95^{b}	***
D-fructose (g/L)						
Must	93.52 ± 8.29^{a}	93.52 ± 8.29^{a}	93.52 ± 8.29^{a}	93.52 ± 8.29^{a}	93.52 ± 8.29^{a}	n.s.
1d	77.36 ± 6.49^{ab}	85.15 ± 5.71^{a}	86.12 ± 12.24^{a}	61.87 ± 7.16^{b}	92.46 ± 5.39^{a}	**
3d	69.52 ± 7.34^{ab}	67.64 ± 2.64^{ab}	68.26 ± 8.09^{ab}	50.38 ± 7.10^{b}	86.10 ± 5.75^{a}	**
6d	41.01 ± 2.30^{b}	63.18 ± 9.20^{ab}	66.49 ± 12.33^{ab}	41.77 ± 7.33^{b}	74.30 ± 13.76^{a}	**
12d	19.47 ± 0.95^{b}	59.26 ± 9.76^{a}	19.64 ± 2.70^{b}	25.27 ± 4.00^{b}	64.93 ± 7.75^{a}	***
End AF	4.08 ± 0.61^{c}	40.76 ± 7.37^{b}	1.05 ± 0.11^{c}	2.65 ± 0.39^{c}	56.12 ± 2.04^{a}	***
Acetic acid (g/L)						
Must	0.02 ± 0.00^{a}	0.02 ±0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	n.s.
1d	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}	0.02 ± 0.00^{a}	n.s.
3d	0.07 ± 0.00^{c}	0.09 ± 0.01^{bc}	0.12 ± 0.01^{b}	0.27 ± 0.03^{a}	0.02 ± 0.00^{d}	***
6d	0.21 ± 0.02^{a}	0.23 ± 0.02^{a}	0.21± 0.02^{a}	0.25 ± 0.02^{a}	0.07 ± 0.01^{b}	***
12d	0.26 ± 0.05^{a}	0.30 ± 0.03^{a}	0.30 ± 0.02^{a}	0.25 ± 0.03^{a}	0.16 ± 0.01^{b}	**
End AF	0.31± 0.05^{c}	0.54 ± 0.06^{b}	0.39 ± 0.04^{c}	0.29 ± 0.02^{c}	0.71 ± 0.06^{a}	***
Total nitrogen (mg/L)						
Must	105.12 ± 5.11^{a}	105.12 ± 5.11^{a}	105.12 ± 5.11^{a}	105.12 ± 5.11^{a}	105.12 ± 5.11^{a}	n.s.
1d	82.54 ± 10.87^{ab}	92.36 ± 3.41^{a}	93.92 ± 12.65^{a}	63.96 ± 9.25^{b}	99.93 ± 6.81^{a}	**
3d	63.81 ± 6.77^{bc}	89.24 ± 13.03^{a}	83.41 ± 0.67^{ab}	46.89 ± 4.87^{c}	91.42 ± 8.74^{a}	***
6d	31.34 ± 2.72^{b}	71.5 ± 10.84^{a}	20.29 ± 3.36^{b}	32.89 ± 3.39^{b}	78.17 ± 9.77^{a}	***
12d	83.06 ± 8.04^{b}	130.56 ± 16.99^{a}	78.90 ± 5.70^{b}	76.40 ± 10.44^{b}	138.32 ± 25.12^{a}	**
End AF	25.15 ± 3.16^{cd}	124.06 ± 15.28^{a}	50.34 ± 3.74^{bc}	19.56 ± 1.91^{d}	71.48 ± 15.83^{b}	***
Glicerol (g/L)						
Must	0.21 ± 0.02^{a}	0.21 ± 0.02^{a}	0.21 ± 0.02^{a}	0.21 ± 0.02^{a}	0.21 ± 0.02^{a}	n.s.
1d	0.50 ± 0.04^{b}	0.41 ± 0.07^{b}	0.40 ± 0.04^{b}	0.61 ± 0.05^{a}	0.26 ± 0.03^{c}	***
3d	1.43 ± 0.07^{b}	1.30 ± 0.07^{b}	1.62 ± 0.15^{b}	2.35 ± 0.28^{a}	0.26 ± 0.04^{c}	***
6d	2.58 ± 0.43^{b}	2.98 ± 0.26^{b}	4.07 ± 0.28^{a}	2.78 ± 0.39^{b}	0.15 ± 0.03^{c}	***
12d	4.68 ± 0.54^{b}	5.38 ± 0.62^{ab}	6.02 ± 0.55^{a}	4.19 ± 0.49^{b}	0.78 ± 0.08^{c}	***
End AF	6.20 ± 0.53^{ab}	5.46 ± 0.11^{bc}	7.25 ± 0.66^{a}	4.31 ± 0.14^{c}	4.79 ± 0.50^{bc}	***
Ethanol (v/v)						
End AF	12.37 ± 1.07^{a}	5.35 ± 0.79^{b}	12.29 ± 2.29^{a}	12.29 ± 1.24^{a}	5.31 ± 0.81^{b}	***

Values are expressed as average of three measurements ± standard deviation.
Abbreviations: S.S., statistical significance; n.d., not detected.
Mead fermented by: *S. cerevisiae* SPF21 (TS); *H. uvarum* YGA36 (TH); sequential inoculum with YGA36 and SPF21 (THS); *S. cerevisiae* EC1118 (C1) and un-inoculated trial subjected to spontaneous fermentation (C2).
Data within a line followed by the same letter are not significantly different according to Tukey's test.
Symbols: **, $P < 0.01$; ***, $P < 0.01$; N.S., not significant.

Glycerol content showed the highest value in trial THS (7.25 g/L) and the lowest in trial C1 (4.31 g/L). Even though glycerol values were lower than those commonly registered in wines (Ribérau-Gayon et al., 2006), their concentrations at the end of the AF were comparable to those found by Schwarz et al. (2020) in mead.

4.3.4. Volatile organic compound composition

Forty-two compounds were emitted by meads (Table 2).

They were grouped into six different chemical classes: alcohols, carbonilic compounds, carboxylic acids, esters, aromatic hydrocarbons and monoterpenes hydrocarbons. Based on their chemical structure and origin, esters were further classified in three sub-groups: ethyl esters (EEAs), acetate esters (EAAs) and miscellaneous esters (MEs). The graphical representation of VOCs analysis is shown in Fig. 3.

The hierarchical dendrogram combined with heat map plot showed that yeast strains significantly affected mead VOCs, highlighting the 11 compounds that showed greater variability. Esters and alcohols were the most abundant classes of mead VOCs. Trial C1 showed the largest amount of total esters identified (41.16 ppm), followed by TS (39.30 ppm), THS (30.17 ppm) and TH (26.68 ppm). When mead was subjected to the spontaneous fermentation (trial C2), the esters generated were barely 2.17 ppm. In general, meads processed exclusively with non-*Saccharomyces* strains show low content of ethyl esters (Sottil et al., 2019), probably because of their low content of ethanol. Focussing deeply on the ethyl esters generated, it came out that the most abundant ethyl ester present in C2 was ethyl acetate (65% of the total content of esters), whereas the esters of fatty acids, the most abundant ones in the other samples, are absent. Ethyl acetate was extremely low in C1 (2.5%) and TS (2.95%) and changed considerably between TH (27.25%) and THS (11.90%).

As reported by Roldán et al. (2011), ethyl acetate content is strictly related to acetic acid production during fermentation. In our study, the differences in terms of acetic acid and ethyl acetate could be explained by the use of non-*Saccharomyces* strain, in fact, a similar effect was registered by Matraxia et al. (2021), wherever inoculated *H. uvarum* strain increased both the level of ethyl acetate and acetic acid in beer.

A low acetate content was observed with *Saccharomyces* as starter strains (2.17 ppm in C1 and 2.19 in TS) while higher concentrations were registered in presence of non-*Saccharomyces* strains (10.70 ppm in TH and 5.98 ppm in THS). The most abundant ethyl esters present in the final meads were ethyl octanoate (ethyl caprylate), particularly present when *Saccharomyces* strains were added (20.30 ppm in TS and 16.61 in C1), ethyl decanoate (ethyl caprate) and ethyl dodecanoate, compounds associated with fruity and floral aroma (Pereira et al., 2019). The last two compounds mostly detected in samples fermented by non-*Saccharomyces* strain. Ethyl hexanoate, responsible for pineapple

aroma (Roldán et al., 2011), was found only in meads from trials C1 and THS (4.15 and 2.86 ppm, respectively).

Table 2. Volatile compound concentrations (ppm) in samples of mead.

Ret. Time	Compounds	C1	TH	THS	TS	C2	S.S.
	ΣAlcohols	**2.98±0.09^{bc}**	**2.86±0.08^{c}**	**3.63±0.11^{a}**	**3.20±0.10^{b}**	**1.61±0.04^{d}**	*******
6.40	3-Methyl-1-butanol	2.21±0.07^{a}	1.37±0.04^{b}	2.19±0.07^{a}	2.30±0.07^{a}	0.75±0.02^{c}	***
13.20	1-Hexanol	-	-	-	-	0.42±0.01	-
30.95	Phenyethyl alcohol	0.75±0.02^{c}	1.50±0.04^{a}	1.43±0.04^{a}	0.88±0.03^{b}	0.43±0.01^{d}	***
57.79	2,4-Di-tert-butylphenol	0.02±0.00^{a}		0.01±0.00^{b}	0.01±0.00^{b}	0.02±0.00^{a}	**
	ΣCarbonyl compounds	**0.07±0.00^{c}**	**0.07±0.00^{c}**	**0.12±0.00^{b}**	**0.12±0.00^{b}**	**0.25±0.01^{a}**	*******
17.80	4-Methyl-2-heptanone	-	-	-	-	0.01±0.00	-
22.80	Octanal	-	-	-	-	0.01±0.00	-
29.45	2-Nonanone	0.02±0.00^{b}	-	-	-	0.12±0.00^{a}	***
42.59	4-Propyl benzaldehyde	0.05±0.00^{d}	0.07±0.00^{c}	0.12±0.00^{a}	0.12±0.00^{a}	0.09±0.00^{b}	***
44.04	2-Undecanone	-	-	-	-	0.02±0.00	-
	ΣCarboxylic acids	**0.15±0.00^{a}**	**-**	**-**	**0.08±0.00^{b}**	**-**	*******
22.35	Hexanoic acid	0.03±0.00	-	-	-	-	-
37.09	Octanoic acid	-	-	-	0.08±0.00	-	-
49.79	Decanoic acid	0.12±0.00	-	-	-	-	-
	ΣEsters	**41.16±1.22^{a}**	**26.68±0.78^{c}**	**30.17±0.91^{b}**	**39.30±1.17^{a}**	**2.17±0.05^{d}**	*******
	ΣEEAs	**39.48±1.17^{a}**	**21.76±0.64^{d}**	**27.71±0.84^{c}**	**33.95±1.01^{b}**	**1.63±0.04^{e}**	*******
3.60	Ethyl acetate	0.96±0.03^{d}	5.93±0.18^{a}	3.59±0.11^{b}	1.16±0.03^{cd}	1.42±0.04^{c}	***
9.10	Ethyl butanoate	0.09±0.00^{a}	-	0.07±0.00^{c}	0.08±0.00^{b}	0.04±0.00^{d}	***
22.65	Ethyl caproate (Ethyl hexanoate)	4.15±0.12^{a}	0.81±0.02^{c}	2.86±0.09^{b}	-	-	***
30.00	Ethyl heptanoate	-	0.02±0.00	-	-	-	-
37.69	Ethyl caprylate (Ethyl octanoate)	16.61±0.50^{b}	3.39±0.10^{d}	12.58±0.38^{c}	20.39±0.61^{a}	0.09±0.00^{e}	***
44.29	Ethyl nonanoate	0.05±0.00^{c}	0.09±0.00^{b}	0.04±0.00^{d}	0.11±0.00^{a}	-	***
50.39	Ethyl *trans*-4-decenoate	0.17±0.00^{b}	0.04±0.00^{c}	0.16±0.00^{b}	0.58±0.02^{a}	-	***
51.24	Ethyl caprate (Ethyl decanoate)	14.61±0.44^{a}	6.45±0.19^{c}	6.88±0.21^{c}	9.30±0.28^{b}	0.08±0.00^{d}	***
63.19	Ethyl dodecanoate	2.80±0.08^{b}	4.88±0.15^{a}	1.50±0.05^{d}	2.28±0.07^{c}	-	***
74.13	Ethyl tetradecanoate	0.02±0.00^{c}	0.14±0.00^{a}	0.01±0.00^{d}	0.03±0.00^{b}	-	***
84.13	Ethyl hexadecanoate	0.02±0.00^{b}	0.03±0.00^{a}	0.02±0.00^{b}	0.02±0.00^{b}	-	***
	ΣEAA	**1.21±0.03^{c}**	**4.77±0.14^{a}**	**2.39±0.07^{b}**	**1.03±0.03^{c}**	**0.48±0.01^{d}**	*******
5.55	Propyl acetate	-	-	-	-	0.13±0.00	-
13.65	3-Methyl-1-butyl acetate	1.05±0.03^{c}	3.07±0.09^{a}	1.68±0.05^{b}	0.77±0.02^{d}	0.22±0.01^{e}	***
21.20	3-Methylpentyl acetate	-	-	-	-	-	-
23.55	Hexyl acetate	0.02±0.00^{b}	-	-	-	0.07±0.00^{a}	***
38.34	Octyl acetate (Caprylyl acetate)	-	-	-	-	0.04±0.00	-
41.24	Phenethyl acetate	0.14±0.00^{d}	1.70±0.05^{a}	0.71±0.02^{b}	0.26±0.01^{c}	0.01±0.00^{e}	***
	ΣMEs	**0.47±0.02^{b}**	**0.14±0.00^{c}**	**0.06±0.00^{c}**	**4.31±0.13^{a}**	**0.05±0.00^{c}**	*******
20.30	Isoamyl propanoate	-	0.03±0.00^{b}	-	3.96±0.12^{a}	0.02±0.00^{b}	***
41.14	Isopentyl hexanoate	0.05±0.00^{b}	0.08±0.00^{a}	-	0.08±0.00^{a}	-	***
47.79	Isobutyl octanoate	-	-	-	0.02±0.00	-	-
54.19	Isoamyl octanoate	0.22±0.01^{a}	-	0.03±0.00^{c}	0.19±0.01^{b}	-	***
60.24	Isobutyl decanoate	0.01±0.00	-	-	-	-	-
65.99	Isopentyl decanoate	0.19±0.01^{a}	0.03±0.00^{c}	0.03±0.00^{c}	0.06±0.00^{b}	0.03±0.00^{c}	***
	ΣAromatic hydrocarbons	**0.29±0.01^{b}**	**0.02±0.00^{d}**	**0.23±0.01^{c}**	**0.35±0.01^{a}**	**0.30±0.01^{b}**	*******
13.00	1,3-Dimethyl benzene	0.01±0.00	-	-	-	-	-
14.45	Styrene	0.04±0.00^{b}	-	0.02±0.00^{c}	0.04±0.00^{b}	0.08±0.00^{a}	***
20.05	1,3,5-Trimethyl benzene	0.02±0.00^{b}	0.01±0.00^{c}	0.03±0.00^{a}	0.02±0.00^{b}	0.03±0.00^{a}	***
40.84	1,3-Di-tert-butylbenzene	0.23±0.01^{b}	0.01±0.00^{d}	0.18±0.01^{c}	0.30±0.01^{a}	0.19±0.01^{c}	***
	ΣMonoterpene hydrocarbons	**0.02±0.00^{a}**	**0.03±0.00^{a}**	**0.02±0.00^{a}**	**0.03±0.00^{a}**	**0.02±0.00^{a}**	**n.s.**
24.25	*p*-Cymene	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	n.s.
24.60	Limonene	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	n.s.
29.25	*p*-Cymenene	-	0.01±0.00^{a}	0.01±0.00^{a}	0.01±0.00^{a}	-	n.s.

Values are expressed as mean ± standard deviation.
Abbreviations: S.S., statistical significance.
Mead fermented by: *S. cerevisiae* SPF21 (TS); *H. uvarum* YGA36 (TH); sequential inoculum with YGA36 and SPF21 (THS); *S. cerevisiae* EC1118 (C1) and un-inoculated trial subjected to spontaneous fermentation (C2).
Data within a line followed by the same letter are not significantly different according to Tukey's test.
Symbols: **, P < 0.01; ***, P < 0.01; n.s., not significant; -, not determined.

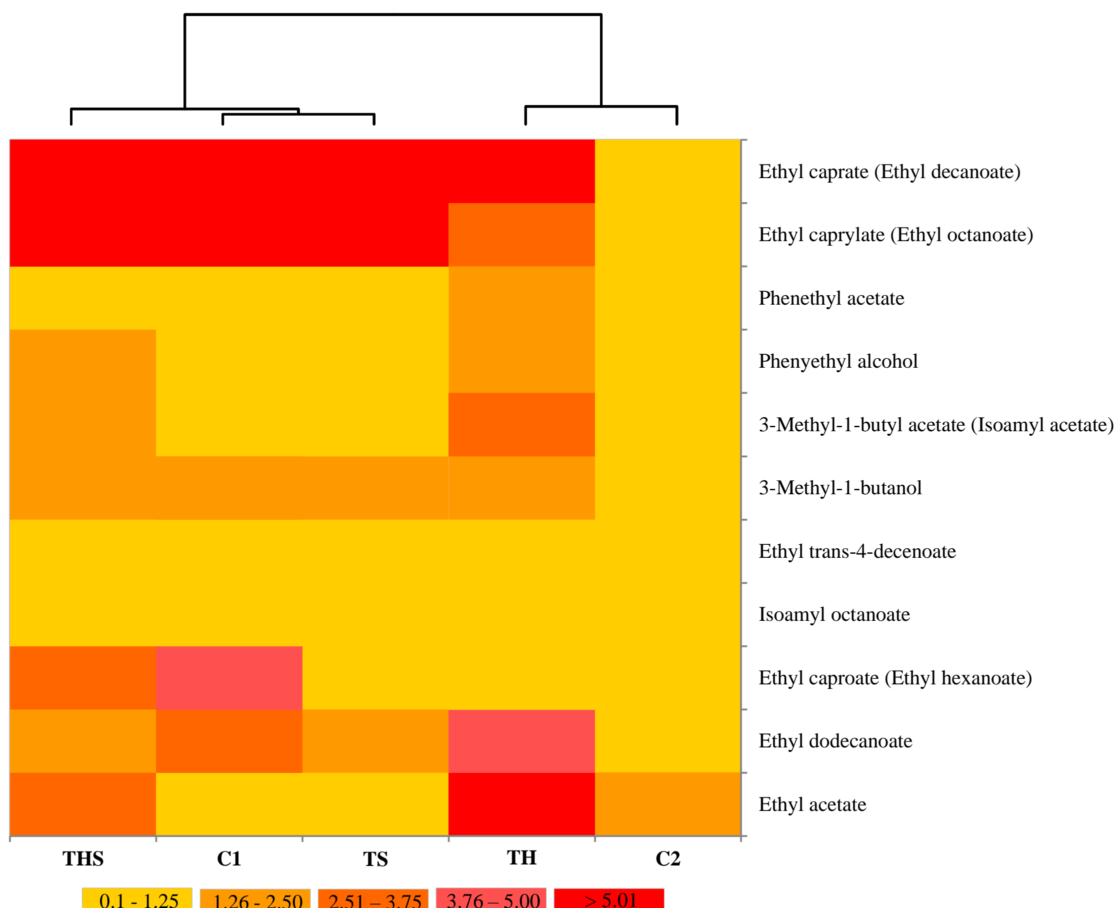


Fig. 3. Distribution of volatile organic compounds among meads. The heat map plot depicts the relative concentration of each VOCs. Mead fermented by: *S. cerevisiae* SPF21 (TS); *H. uvarum* YGA36 (TH); sequential inoculum with YGA36 and SPF21 (THS); *S. cerevisiae* EC1118 (C1) and un-inoculated trial subjected to spontaneous fermentation (C2).

The most abundant alcohol in all samples was 3-methyl-1-butanol (isoamyl alcohol), which is generally found in meads (Roldán et al., 2011). In particular, the amounts registered from the trials inoculated with *Saccharomyces* strains (2.30 ppm in TS, 2.21 in C1 and 2.19 in THS) were consistently higher than those processed with non-*Saccharomyces* strain (1.37 in TH). On the contrary, trial TH generated the highest concentrations of isoamyl acetate (3.07 ppm) and isoamyl propanoate (0.03 ppm).

4.3.5. Sensory analysis

The results of the quantitative sensory analysis are reported in Fig. 4a. All experimental meads showed consistent differences related to the starter *Saccharomyces* and non-*Saccharomyces* strains. One of the major differences was represented by the appearance, evaluated in terms of yellow colour which is related to the phenolic, flavonoid and mineral contents (Pereira et al., 2017), as well as oxidation or condensation reaction products or their adsorption by fermentative yeasts (Mazauric and Salmon, 2005). TS and TH trials showed a light straw-yellow colour, while THS originated a more

gold-yellowish colour mead, confirming that starter strains influence the colour of the final product (Belda et al., 2016; Schwarz et al., 2020).

The quantitative sensory analysis revealed a high heterogeneity between the trials (Fig. 4a). For each trial, two aroma-related attributes showed the highest scores: floral and honey for TH; persistence and overall impression for THS; fruity and vinous for TS; intensity and spicy for C1. The highest scores for the negative attributes (rancid, acetic and oxidized) were displayed by the spontaneously fermented trial (C2). In terms of mead taste, TH was characterized by the highest sweetness, since the AF was not completed, TS by the highest intensity, C1 by the high persistence and acidity, THS by the highest overall impression. C2 taste was the less appreciated due to the several defects encountered. Regarding the overall quality, TS mead resulted the best product. followed by THS and C1 meads. AHC classified the trials in accordance with their mutual dissimilarity (Fig. 4b).

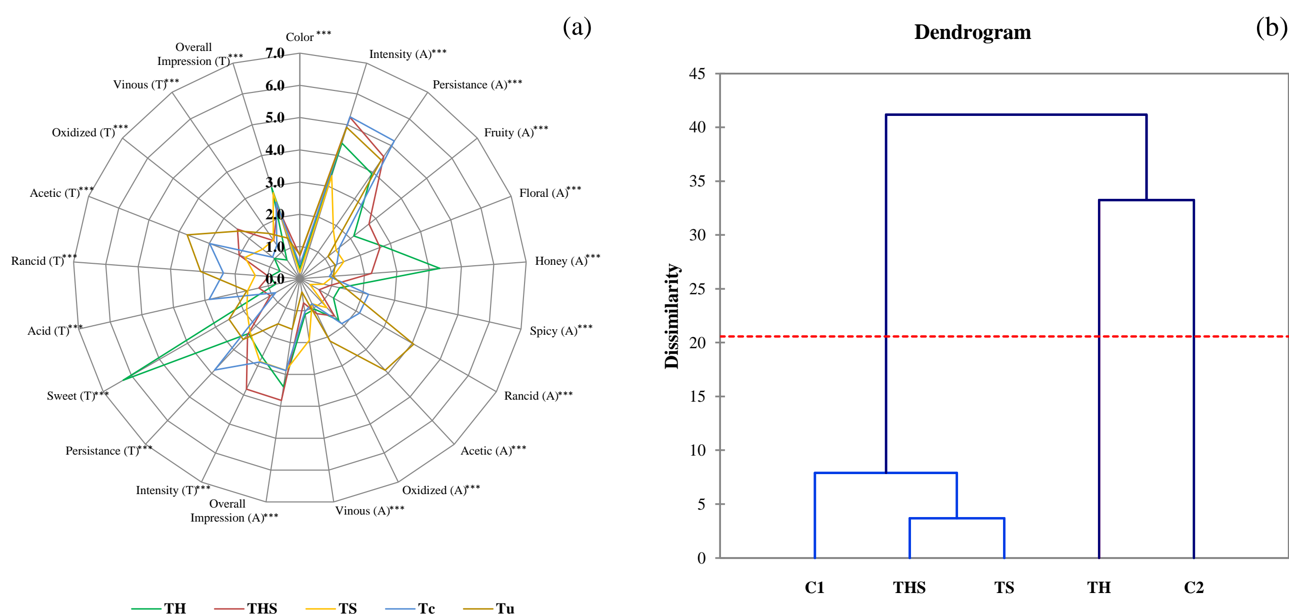


Fig. 4. Sensory analysis performed on meads: (a) spider plot of average scores for appearance, aroma and taste attributes determined by judges during tasting sessions; (b) dendrogram of mead samples resulting from AHC based on values of sensory analysis. Mead fermented by: *S. cerevisiae* SPF21 (TS); *H. uvarum* YGA36 (TH); sequential inoculum with YGA36 [THS(H)] and SPF21 [THS(S)]; *S. cerevisiae* EC1118 (C1) and un-inoculated trial subjected to spontaneous fermentation (C2). Different small letters indicate statistically different overall quality values between trials.
Symbols: ***, $P < 0.01$.

This analysis classified meads using 22 variables on the basis of the results from quantitative sensory analysis. All experimental meads were clearly separated into three clusters considering a dissimilarity of 20.58%. Interestingly, all trials performed with *S. cerevisiae* (TS, THS and C1) grouped in the same cluster. Spontaneous fermented C2 and TH trials showed the highest value of dissimilarity and clustered separately.

4.4. CONCLUSIONS

Two groups of strains belonging to the species *S. cerevisiae* and *H. uvarum*, previously isolated from fermented matrices related to the production of "*Spiritu re fascitrari*", a traditional Sicilian distillate processed from honey by-products, were screened and evaluated for their performance in mead application as starters or co-cultures. Despite the general application of wine and beer related starter strains, in this work, for the first time yeast strains of different genera isolated from honey-related matrices were tested in combination to evaluate their effect on physico-chemical and sensory properties of mead. The different inoculums showed an improved quality of mead in comparison to the production performed with the commercial oenological *S. cerevisiae* EC1118. An absence of off-odours and off-flavours and a better aromatic perception were observed in the experimental trials started with *H. uvarum* co-inoculated with *S. cerevisiae*. Besides the highest alcohol production, *S. cerevisiae* strains determined a high concentration of ethyl esters and a low generation of acetate esters. The most abundant ethyl esters present in these trials were fatty acids esters associated with fruity and floral aroma. In conclusion, the application of *Saccharomyces* and non-*Saccharomyces* strains from honey by-products was proven to be the natural solution to produce high quality mead.

4.5 REFERENCES

Almeida, E.L.M.D., Moreira E Silva, G., Vassalli, I.D.A., Silva, M.S., Santana, W.C., Silva, P H.A.D., Eller, M.R., 2020. Effects of nitrogen supplementation on *Saccharomyces cerevisiae* JP14 fermentation for mead production. Food Sci. Technol. 40, 336-343. https://doi.org/10.1590/fst.11219.

Ameur, L.A., Rega, B., Giampaoli, P., Trystram, G., Birlouez-Aragon, I., 2008. The fate of furfurals and other volatile markers during the baking process of a model cookie. Food Chem. 111(3), 758-763. http://dx.doi.org/10.1016/j.foodchem.2007.12.062.

Aponte, M., Blaiotta, G., 2016. Potential role of yeast strains isolated from grapes in the production of Taurasi DOCG. Front. Microbiol. 7, 809. https://dx.doi.org/10.3389%2Ffmicb.2016.00809.

Araújo, T.M., Souza, M.T., Diniz, R.H.S., Yamakawa, C.K., Soares, L.B., Lenczak, J.L., Oliveira, J.V.D.C., Goldman, G.H., Barbosa, E.A., Campos, A.C.S., Brandão R.L., Castro, I.M., 2018. Cachaça yeast strains: alternative starters to produce beer and bioethanol. Antonie Van Leeuwenhoek 111(10), 1749-1766. https://doi.org/10.1007/s10482-018-1063-3.

Barry, J.P., Metz, M.S., Hughey, J., Quirk, A., Bochman, M.L. (2018). Two novel strains of *Torulaspora delbrueckii* isolated from the honey bee microbiome and their use in honey fermentation. Fermentation 4(2), 22. https://doi.org/10.3390/fermentation4020022.

Bednarek, M., Szwengiel, A., Flórez, A.B., Czarnecki, Z., Mayo, B., 2019. Effect of different starter cultures on chemical and microbial parameters of buckwheat honey fermentation. Food Microbiol. 82, 294-302.

Belda, I., Conchillo, L.B., Ruiz, J., Navascués, E., Marquina, D., Santos, A., 2016. Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking. Int. J. Food Microbiol. 223, 1-8.

Capece, A, Fiore, C, Maraz, A, Romano, P., 2005. Molecular and technological approaches to evaluate strain biodiversity in *Hanseniaspora uvarum* of wine origin. J. Appl. Microbiol. 98, 136-144.

De Rubeis, M.G., 2016. Idromele. Miti, storia e preparazioni della bevanda degli dei. Ed. I Doni delle Muse, Italy.

Domizio, P., Romani, C., Lencioni, L., Comitini, F., Gobbi, M., Mannazzu, I., Ciani, M., 2011. Outlining a future for non-*Saccharomyces* yeasts: selection of putative spoilage wine strains to be used in association with *Saccharomyces cerevisiae* for grape juice fermentation. Int. J. Food Microbiol. 147(3), 170-180.

Elhalis, H., Cox, J., Frank, D., Zhao, J., 2021. Microbiological and biochemical performances of six yeast species as potential starter cultures for wet fermentation of coffee beans. LWT 137, 110430.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Int. J. Syst. Evol. Microbiol. 49(1), 329-337.

Fernández-Espinar, M. T., Esteve-Zarzoso, B., Querol, A., Barrio, E., 2000. RFLP analysis of the ribosomal internal transcribed spacers and the 5.8 S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of flor yeasts. Antonie van Leeuwenhoek 78(1), 87-97.

Francesca, N., Chiurazzi, M., Romano, R., Aponte, M., Settanni, L., Moschetti, G., 2010. Indigenous yeast communities in the environment of "Rovello bianco" grape variety and their use in commercial white wine fermentation. World J. Microbiol. Biotechnol. 26(2), 337-351.

Gaglio, R., Alfonzo, A., Francesca, N., Corona, O., Di Gerlando, R., Columba, P., Moschetti, G., 2017. Production of the Sicilian distillate "*Spiritu re fascitrari*" from honey by-products: An interesting source of yeast diversity. Int. J. Food Microbiol. 261, 62-72.

Gibson, B.R., 2011. 125th anniversary review: improvement of higher gravity brewery fermentation via wort enrichment and supplementation. J. Inst. Brew. 117(3), 268-284.

Granchi, L., Bosco, M., Messini, A., Vincenzini, M., 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR–RFLP analysis of the rDNA ITS region. J. Appl. Microbiol. 87(6), 949-956.

Gschaedler, A., 2017. Contribution of non-conventional yeasts in alcoholic beverages. Curr. Opin. Food Sci. 13, 73-77.

Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J., Querol, A., 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. Arch. Microbiol. 169(5), 387-392.

Gupta, J.K., Sharma, R. 2009. Production technology and quality characteristics of mead and fruit-honey wines: A review. Indian J. Nat. Prod. Resour. 8, 345-355.

Hernández, C., Serrato, J.C., Quicazan, M., 2015. Evaluation of physicochemical and sensory aspects of mead, produced by different nitrogen sources and commercial yeast. Chem. Eng. Trans. 43, 1-6.

Holt, S., Mukherjee, V., Lievens, B., Verstrepen, K.J., Thevelein, J.M., 2018. Bioflavoring by non-conventional yeasts in sequential beer fermentations. Food Microbiol. 72, 55-66.

Hong, Y.A., Park, H.D., 2013. Role of non-*Saccharomyces* yeasts in Korean wines produced from Campbell Early grapes: potential use of *Hanseniaspora uvarum* as a starter culture. Food Microbiol. 34(1), 207-214.

Jackson, R.S. 2016. Wine tasting. A professional handbook, third edition. Eds. Cool Climate enology and Viticulture Institute, Brock University, St. Catharines, Ontario, Canada.

Jiranek, V., Langridge, P., Henschke, P.A., 1995. Regulation of hydrogen sulfide liberation in wine-producing *Saccharomyces cerevisiae* strains by assimilable nitrogen. Appl. Environ. Microbiol. 61(2), 461-467.

Legras, J., Karst, F., 2003. Optimisation of interdelta for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiol. Lett. 221, 249-255.

Li, R., Sun, Y., 2019. Effects of honey variety and non-*Saccharomyces cerevisiae* on the flavor volatiles of mead. J. Am. Soc. Brew. Chem. 77(1), 40-53.

Martorana, A., Alfonzo, A., Gaglio, R., Settanni, L., Corona, O., La Croce, F., Vagnoli, P., Caruso, T., Moschetti, G., Francesca, N., 2017. Evaluation of different conditions to enhance the performances of *Lactobacillus pentosus* OM13 during industrial production of Spanish-style table olives. Food Microbiol. 61, 150-158.

Matraxia, M., Alfonzo, A., Prestianni, R., Francesca, N., Gaglio, R., Todaro, A., Alfeo, V., Perretti, G., Columba, P., Settanni, L., Moschetti, G., 2021. Non-conventional yeasts from fermented honey by-products: Focus on *Hanseniaspora uvarum* strains for craft beer production. Food Microbiol. 99,103806.

Mazauric, J.P., Salmon, J.M., 2005. Interactions between yeast lees and wine polyphenols during simulation of wine aging: I. Analysis of remnant polyphenolic compounds in the resulting wines. J. Agric. Food Chem. 53(14), 5647-5653.

Mazzei, P., Francesca, N., Moschetti, G., Piccolo, A., 2010. NMR spectroscopy evaluation of direct relationship between soils and molecular composition of red wines from Aglianico grapes. Anal. Chim. Acta 673(2), 167-172.

Mendes-Ferreira, A., Mendes-Faia, A., Leao, C., 2002. Survey of hydrogen sulphide production by wine yeasts. J. Food Prot. 65(6), 1033-1037.

Parrini, M., 2021. I colori dell'idromele. Come produrre i tuoi idromeli, melomeli, braggot e pazzomeli fatti in casa. Edizioni LSWR, Italy.

Pasteur, L., 1858. Nouveaux faits concernant l’histoire de la fermentation alcoolique. C. R. Chim. 47, 1011-1013.

Pereira, A.P., Mendes-Ferreira, A., Dias, L.G., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2019. Volatile composition and sensory properties of mead. Microorganisms 7(10), 404.

Pereira, A.P., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A., 2015b. Improvement of mead fermentation by honey-must supplementation. J. Inst. Brew. 121(3), 405-410.

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2013. High-cell-density fermentation of *Saccharomyces cerevisiae* for the optimisation of mead production. Food Microbiol. 33(1), 114-123.

Pereira, A.P., Mendes-Ferreira, A., Oliveira, J.M., Estevinho, L.M., Mendes-Faia, A., 2015a. Mead production: effect of nitrogen supplementation on growth, fermentation profile and aroma formation by yeasts in mead fermentation. J. Inst. Brew. 121(1), 122-128.

Pereira, A.P., Oliveira, J.M., Mendes-Ferreira, A., Estevinho, L.M., Mendes-Faia, A., 2017. Mead and other fermented beverages. In *Current developments in biotechnology and bioengineering* (pp. 407-434). Elsevier.

Platt, D., 1988. Memoire sur la fermentation appelée lactique (Louis Pasteur). Del. Med. J. 60(1), 23-24.

Qureshi, N., Tamhane, D.V., 1987. Production of mead by immobilized cells of *Hansenula anomala.* Appl. Microbiol. Biotechnol. 27(1), 27-30.

Ramalhosa, E., Gomes, T., Pereira, A.P., Dias, T., Estevinho, L.M., 2011. Mead production: Tradition versus modernity, in: Advances in food and nutrition research (Vol. 63, pp. 101-118). Academic Press.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2006. Handbook of enology*,* Volume 1: The microbiology of wine and vinifications. John Wiley & Sons, Chichester.

Rogers, A., 2014. Proof: the science of booze. Houghton Mifflin Harcourt, Boston.

Roldán, A., Van Muiswinkel, G. C. J., Lasanta, C., Palacios, V., Caro, I., 2011. Influence of pollen addition on mead elaboration: Physicochemical and sensory characteristics. Food Chem. 126(2), 574-582.

Schwarz, L.V., Marcon, A.R., Delamare, A.P.L., Agostini, F., Moura, S., Echeverrigaray, S., 2020. Selection of low nitrogen demand yeast strains and their impact on the physicochemical and volatile composition of mead. J. Food Sci. Technol. 57(8), 2840-2851.

Sottil, C., Salor-Torregrosa, J. M., Moreno-Garcia, J., Peinado, J., Mauricio, J. C., Moreno, J., Garcia-Martinez, T., 2019. Using *Torulaspora delbrueckii*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus* wine yeasts as starter cultures for fermentation and quality improvement of mead. Eur. Food Res. Technol. 245(12), 2705-2714.

Sroka, P., Tuszyński, T., 2007. Changes in organic acid contents during mead wort fermentation. Food Chem. 104(3), 1250-1257.

Tristezza, M., Tufariello, M., Capozzi, V., Spano, G., Mita, G., Grieco, F., 2016. The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. Front. Microbiol. 7, 670.

Ukpabi, U.J., 2006. Quality evaluation of meads produced with cassava (*Manihot esculenta*) floral honey under farm conditions in Nigeria. Trop. Subtrop. Agroecosyst. 6(1), 37-41.

Varela, C., 2016. The impact of non-*Saccharomyces* yeasts in the production of alcoholic beverages. Appl. Microbiol. Biotechnol. 100(23), 9861-9874.

Vilanova, M., Ugliano, M., Varela, C., Siebert, T., Pretorius, I.S., Henschke, P.A., 2007. Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Appl. Microbiol. Biotechnol. 77(1), 145-157.

Wang, C., Mas, A., Esteve-Zarzoso, B., 2015. Interaction between *Hanseniaspora uvarum* and *Saccharomyces cerevisiae* during alcoholic fermentation. Int. J. Food Microbiol. 206, 67-74.

CHAPTER 5

General conclusions

This PhD thesis presented different approaches to improve fermented beverages overall quality. The research of fermenting microorganisms capable of improving the microbiological, physical, chemical, sensory and organoleptic properties of fermented alcoholic beverages such as wine, beer, mead, cider, is considered today a key point for the development of the sector. Through an in-depth characterization of the microbial ecology of high-sugar matrices, it was possible to isolate, characterize and apply different yeast strains.

For the first time, a selected strain of *H. uvarum* isolated from fermented honey by-products was applied in beer wort fermentation, in different inoculum combinations with US-05 *S. cerevisiae* commercial brewing strain. After a deep characterization of strains for their brewing properties (ethanol tolerance, hop resistance, cross resistance, flocculation, hydrogen sulphide production, growth kinetics), the best strain was used in beer production. Interestingly, the use of this non-*Saccharomyces* strain improved the sensorial properties of final beers, when used sequentially with US-05. A dominance of *H. uvarum* was registered when the pitching ratio was 10:1 respect to *S. cerevisiae*. The differences in terms of chemical and volatile compounds and on sensory analysis where highlighted when differences between various inoculation techniques and cultures concentrations were employed.

In a second study, the *S. cerevisiae* ecology of "*Spiritu re Fascitrari*" distillate was deeply characterized under genotypical and technological aspects. From over six hundred isolates, a set of 14 strains was tested in micro-fermentations of honey must and the three best strains were further applied to ferment a decoction of honey by-product, improving the sensorial and microbiological quality of the final distillates.

Finally, the two microbial groups employed in the former studies (*H. uvarum* and *S. cerevisiae*), both isolated from honey sector, where sequentially used in mead fermentation. The results of single strain and combined inoculum highlighted that the use of these strains could strongly affect the final product characteristics. Mead fermented by *H. uvarum* resulted particularly sweet, since an incomplete alcoholic fermentation occurred, resulting appreciated by some sensory judges. The combined use of *H. uvarum* and *S. cerevisiae* strains, generated a more balanced mead, characterized by the highest score for aroma persistence and overall impression.

This doctoral thesis highlighted how the use of unconventional yeasts can represent a promising strategy for the quality improvement of fermented beverages. In the footsteps of the wine sector, where the use of these microorganisms represents a consolidated reality, the use of novel yeasts can lead to a better shaping of the aromatic profiles of fermented beverages, also modulating the inoculation methods of the selected strains.

The study of microbial ecology of territorial and traditional matrices, can represent an important source of microorganisms potentially employable to characterize and distinguish fermented beverages on a rapidly expanding market. This approach not only fosters the consumer's thirst for novelty but can have a positive impact on the profitability of companies in the sector.

CHAPTER 6

List of publications

Diversity of *Saccharomyces cerevisiae* strains associated to racemes of Grillo grape variety

Alfonzo A.[a], Francesca N.[a] Francesca N., **Matraxia M.[a]**, Craparo V.[a], Naselli V.[b], Mercurio V.[c], Moschetti G.[a]

[a] Department of Agricultural, Food and Forestry Science, University of Palermo, Viale delle Scienze 4, Palermo, 90128, Italy.

[b] Cantine Europa Società Cooperativa Agricola, SS 115 Km 42.400, Petrosino, 91020, Italy.

[c] Le Ali di Mercurio s.r.l., Vincenzo Mercurio Wine Consultant, Castellammare di Stabia, Napoli, 80053, Italy.

FEMS Microbiology Letters Vol. 367, Issue 12/2021 Article number fnaa079

ABSTRACT

The most important oenological characteristics of high-quality sparkling wines are high content of acidity and low pH. Racemes are late-maturing grapes of Grillo variety characterized by low pH and high content of tartaric and malic acids and, due to their intrinsic characteristics, might represent an interesting technological solution to increase acid quality of base sparkling wine. To this purpose, the use of yeasts able to ferment grape must at very low pH is mandatory for the success of the process. In this work, 261 *Saccharomyces cerevisiae* isolated from spontaneous vinifications of Grillo grape racemes were subject to intraspecific characterization by interdelta analysis which evidenced a total population consisting of 82 strains which were screened for their basis of technological traits including SO_2 and alcohol tolerance, flocculence, growth at low temperatures and qualitative features such as H_2S production. A total of 11 strains with interesting technological performance in vitro were inoculated into musts obtained from racemes of Grillo grape variety and micro-fermentation were monitored. For the first time an ecological investigation of yeast associated to raceme grapes has been carried out and provided an innovative strategy to improve the acidity of a Sicilian sparkling base wine from Grillo grape variety.

Monitoring commercial starter culture development in presence of red grape pomace powder to produce polyphenol-enriched fresh ovine cheeses at industrial scale level

Barbaccia P.[a], Busetta G.[a], **Matraxia M.[a]**, Sutera A.M.[b], Craparo V.[a], Moschetti G.[a], Francesca N.[a], Settanni L.[a], Gaglio R.[a]

[a] Dipartimento Scienze Agrarie, Alimentari e Forestali, Ed. 5, Università degli Studi di Palermo, Viale delle Scienze, Palermo, 90128, Italy

[b] Dipartimento Scienze Veterinarie, University of Messina, Messina, 98168, Italy

Fermentation *Open Access* Vol. 7, Issue 1 March 2021 Article number 35

ABSTRACT

Red grape Nero d'Avola cultivar grape pomace powder (GPP) was applied during fresh ovine cheese production in order to increase polyphenol content. Before cheeses were produced, the bacteria of a freeze-dried commercial starter culture were isolated and tested in vitro against GPP. Two dominant strains, both resistant to GPP, were identified. The starter culture was inoculated in pasteurized ewe's milk and the curd was divided into two bulks, one added with 1% (w/w) GPP and another one GPP-free. GPP did not influence the starter culture development, since lactic acid bacteria (LAB) counts were 10^9 CFU/g in both cheeses at 30 d. To exclude the interference of indigenous LAB, the pasteurized milk was analyzed, and several colonies of presumptive LAB were isolated, purified and typed. Four strains were allotted into *Enterococcus* and *Lacticaseibacillus* genera. The direct comparison of the polymorphic profiles of cheese bacteria evidenced the dominance of the starter culture over milk LAB. The addition of GPP increased cheese total phenolic compounds by 0.42 g GAE/kg. Sensory evaluation indicated that GPP-enriched cheese was well appreciated by the judges, providing evidence that GPP is a suitable substrate to increase the availability of total phenolic content in fresh ovine cheese.

Sourdough “ciabatta” bread enriched with powdered insects: Physicochemical, microbiological, and simulated intestinal digesta functional properties

Gaglio R.[a], Barbera M.[a], Tesoriere L.[b], Osimani A.[c], Busetta G.[a], **Matraxia M.[a]**, Attanzio A.[b], Restivo I.[b], Aquilanti L.[c], Settanni L.[a]

[a] Dipartimento Scienze Agrarie, Alimentari e Forestali, Università degli Studi di Palermo, Viale delle Scienze 4, Palermo, 90128, Italy

[b] Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Università degli Studi di Palermo, Via Archirafi 34, Palermo, 90123, Italy

[c] Dipartimento Scienze Agrarie, Alimentari e Ambientali, Università Politecnica delle Marche, via Brecce Bianche, Ancona, 60131, Italy

Innovative Food Science and Emerging Technologies Vol. 72, August 2021, Article number 102755

ABSTRACT

Powdered mealworm (MW) and buffalo worm (BW) larvae were used to functionalize sourdough Italian-style breads. Sourdough inoculum was started with *Levilactobacillus brevis*, *Weissella cibaria* and *Leuconostoc citreum*. The doughs were SBS (semolina plus powdered BW larvae and sourdough) and SMS (semolina plus powdered MW larvae and sourdough) whose pHs (4.32 and 4.21, respectively) were higher than that of control (3.81). The highest fermentation quotient (lactate/acetate molar ratio) was recorded in SMS (4.46). LAB reached viable counts of about 10^9 CFU g^{-1} in almost all doughs. Insects impacted bread VOCs with dodecanal, 2.4-dodecadienal and 2-octenal-2-butyl. SBS and SMS increased the antioxidant capacity of breads by 42 and 69%, respectively. SMS decreased the glucose release in the bio-accessible fraction by 70% reducing the glycemic index of bread. Control breads were more appreciated by the sensory panel than insect-containing breads, though SMS breads were characterized by a slightly lower overall assessment.

Non-conventional yeasts from fermented honey by-products: Focus on *Hanseniaspora uvarum* strains for craft beer production

Matraxia M.[a], Alfonzo A.[a], Prestianni R.[a], Francesca N.[a], Gaglio R.[a], Todaro A.[a], Alfeo V.[b], Perretti G.[b], Columba P.[a], Settanni L.[a], Moschetti G.[a]

[a] Dipartimento Scienze Agrarie, Alimentari e Forestali, Università degli Studi di Palermo, Viale delle Scienze 4, Palermo, 90128, Italy

[b] Italian Brewing Research Centre, Department of Agricultural, Food and Environmental Science, University of Perugia, Perugia, 06126, Italy

Food Microbiology Vol. 99, October 2021, Article number 103806

ABSTRACT

The increasing interest in novel beer productions focused on non-*Saccharomyces* yeasts in order to pursue their potential in generating ground-breaking sensory profiles. Traditional fermented beverages represent an important source of yeast strains which could express interesting features during brewing. A total of 404 yeasts were isolated from fermented honey by-products and identified as *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii* and *Hanseniaspora uvarum*. Five *H. uvarum* strains were screened for their brewing capability. Interestingly, *H. uvarum* strains showed growth in presence of ethanol and hop and a more rapid growth than the control strain *S. cerevisiae* US-05. Even though all strains showed a very low fermentation power, their concentrations ranged between 7 and 8 Log cycles during fermentation. The statistical analyses showed significant differences among the strains and underlined the ability of YGA2 and YGA34 to grow rapidly in presence of ethanol and hop. The strain YGA34 showed the best technological properties and was selected for beer production. Its presence in mixed- and sequential-culture fermentations with US-05 did not influence attenuation and ethanol concentration but had a significant impact on glycerol and acetic acid concentrations, with a higher sensory complexity and intensity, representing promising co-starters during craft beer production.

Effects of different yeast strains, nutrients and glutathione-rich inactivated yeast addition on the aroma characteristics of Catarratto wines

Alfonzo A.[a], Prestianni R.[a], Gaglio R.[a], **Matraxia M.[a]**, Maggio A.[b], Naselli V.[a], Craparo V.[a], Badalamenti N.[b], Bruno M.[b], Vagnoli P.[c], Settanni L.[a], Moschetti G.[a]

[a] Department of Agricultural, Food and Forest Science, University of Palermo, Viale delle Scienze 4, Palermo, 90128, Italy

[b] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze, Parco d'Orleans II, building 17, Palermo, Italy

[c] Lallemand Italia, Via Rossini 14/B, Castel D'Azzano, 37060, VR, Italy

International Journal of Food Microbiology Vol. 360, 16 December 2021, Article number 109325

Abstract

Catarratto is one of the most common non-aromatic white grape varieties cultivated in Sicily (Southern Italy). In order to improve the aromatic expression of Catarratto wines a trial was undertaken to investigate the effect of yeast strain, nutrition and reduced glutathione. Variables included two *Saccharomyces cerevisiae* strains, an oenological strain (GR1) and one isolated from honey by-products (SPF52), three different nutrition regimes (Stimula Sauvignon Blanc™ (SS), Stimula Chardonnay™ (SC) and classic nutrition practice), and a specific inactivated yeast rich in reduced glutathione to prevent oxidative processes [Glutastar™ (GIY)] ensuing in ten treatments (T1-T10). Microbiological and chemical parameters demonstrated the aptitude of strain SPF52 to successfully conduct alcoholic fermentation. During fermentation, the Saccharomyces yeast populations ranged from 7 to 8 logarithmic CFU/mL. All wines had a final ethanol content ranging between 12.91 and 13.85% (v/v). The dominance of the two starter strains over native yeast populations was higher than 97% as estimated by interdelta analysis. The addition of nutrients SS or SC increased the aromatic complexity of the wines as reflected by volatile organic compounds (VOCs) composition and sensory profiles. In particular, 32 VOCs were identified; alcohols (62.46–81.1%), thiols (0.27–0.87%), ethers (0.09–0.16%), aldehydes (0–1.21%), ketones (0–2.28%), carboxylic acids (4.21–12.32%), esters (0–10.85%), lactones (0.9–1.49%) and other compounds (0.77–6.9%). Sensory analysis demonstrated a significant impact on wine aroma in relation to yeast starter strain used, the type of nutrition (SS, SC or classic nutrition) and the presence/absence of GIY. The wines produced with GR1 yeast strain and SS (T2), SPF52 with SC (T9) both in presence of GIY showed higher overall quality. Trials T2 and T9 showed the highest scores for 13 and 18 attributes, respectively. The different nutrition, addition of GIY and the yeast starter strains diversified and enhanced sensory expression of Catarratto wines.

Technological screening and application of *Saccharomyces cerevisiae* strains isolated from fermented honey by-products for the sensory improvement of *Spiritu re fascitrari*, a typical Sicilian distilled beverage

Francesca N.[a], Gaglio R.[a], **Matraxia M.[a]**, Naselli V.[a], Prestianni R.[a], Settanni L.[a], Badalamenti N.[b], Columba P.[a], Bruno M.[b], Maggio A.[b], Alfonzo A.[a], Moschetti G.[a]

[a] Department of Agricultural, Food and Forest Science, University of Palermo, Viale delle Scienze 4, Palermo, 90128, Italy

[b] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze, Parco d'Orleans II, Building 17, Palermo, Italy

Food Microbiology Vol. 104, June 2022, Article number 103968

ABSTRACT

"*Spiritu re fascitrari*" is a Sicilian alcoholic beverage obtained through distillation of a decoction of spontaneously fermented honey by-products (FHP). The production process often leads to sensorial defects due to the unstable alcoholic fermentation. The objective of this work was to select *Saccharomyces cerevisiae* strains from FHP to be used as starter in decoction fermentation. Based on chemical, microbiological and technological data, from a total of 91 strains three *S. cerevisiae* were selected for further testing to produce FHP at laboratory scale level. After FHP distillation, the analysis of volatile organic compounds showed a complex mixture of sensory active molecules, mainly alcohols and aldehydes. Among the alcohols, 3-methyl-1-butanol, 2-methyl-1-butanol, phenylethyl alcohol, hexadecanol and octadecanol were found at the highest concentrations. Among the carboxylic acids, acetic acid was mainly detected in the spontaneously fermented samples. FHP fermented with the three selected strains were not characterized by the presence of off-odours or off-flavours. The results obtained in this work demonstrate that the selected *S. cerevisiae* strains are promising starters to stabilize the production of distilled alcoholic beverages produced from honey by-products.

Publications on national journals

- **Matraxia M.**, Ciminata A., Alfonzo A., Moschetti G., Francesca N. (2019). Le proprietà sensoriali delle birre artigianali legate ai microrganismi della fermentazione. Mensile Agrisicilia, anno X, n. 1-2 gennaio-febbraio 2019.
- Paternostro, R., **Matraxia, M.**, Cascio, M., Ricci, M., Dabove, L., Camaschella, A., Moschetti, G. (2019). Ricerca di lieviti autoctoni per migliorare il profilo aromatico delle birre artigianali. Mensile Agrisicilia, anno X, n. 10, ottobre 2019.
- N. Francesca, R. Prestianni, **M. Matraxia**, V. Naselli, V. Craparo, A. Maggio, N. Badalamenti, P. Vagnoli, S. Lo Voi, V. Mercurio, G. Moschetti, A. Alfonzo (2021). La nutrizione microbica esalta la complessità aromatica. Vite & Vino Vol. 3, pag.48.
- N. Francesca, G. Spanò, V. Naselli, **M. Matraxia**, R. Prestianni, S. Lo Voi, L. Turano, D. Micciché, A. Sacco, A. Pisciotta, G. Moschetti, A. Alfonzo, A. Pisciotta (2021) La base vino adatta per spumanti di qualità. Vite & Vino Vol. 4, pag. 60.

A tutte le persone che mi sono state vicine

durante questo percorso di vita

e di crescita personale e professionale.